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The normal operating range of soil functioning

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The normal operating range of soil functioning

Understanding the natural fluctuations of nitrogen cycling communities

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"There are no shortcuts to any place worth going"

Beverly Sills

Contents

Chapter 1

General introduction

Michele C Pereira e Silva

Soil represents a highly heterogeneous and dynamic environment for its microbiota. The different components of the solid fractions in soil provide a range of different microhabitats (van Elsas and Trevors, 2006), promoting the development and maintenance of an extremely large number of niches (Tiedje *et al.*, 2001; Ettema and Wardle 2002). These niches have a direct effect on the living fraction of soil, whose biodiversity (number of different organism types and their relative abundance) is uncountable. Soil microorganisms are the key drivers of the life support functions of soil (LSF), including nutrient acquisition (Sprent, 2001), nitrogen cycling (Kowalchuk and Stephen, 2001), carbon cycling (Hogberg *et al.*, 2001) and soil formation (Rilling and Mummey 2006). Soil microbial communities are important in maintaining the quality of both natural and agriculturally managed soil systems. Moreover, microorganisms are highly responsive to environmental influences (disturbances), such as those incurred by abiotic (temperature, pH, soil moisture and soil structural or textural type) and biotic factors (the composition and diversity of the microbial community), such as soil type, nutrient status, pH, and moisture (Girvan *et al.*, 2003; Gelsomino *et al.*, 1999; Lauber *et al.*, 2009). These responses to (a)biotic factors are likely to have an effect on functions driven by the soil microbiota (Figure 1.1).

Temperate-climate countries like the Netherlands have a successful history of agriculture, which relates to the favorable conditions for crop growth. However, a sound fundamental knowledge of the impact of crop type, agricultural management regime and putative stressors on soil functioning is actually lacking. In order to understand the relevance of crop/ management/ stressor-induced changes, the natural variation of soil function caused by crop, management as well as climatic effects needs to be addressed in the context of the so-called normal operating range (NOR) of (agricultural) soil. The resulting description of the dynamic soil status will provide a background against which out-of-range situations can be compared (Kowalchuk *et al.*, 2003; Bruinsma *et al.*, 2003).

Potential biological indicators of soil quality

The applicability of the NOR of soils as a monitoring tool, depends strongly on its ability to detect disturbances, as well as its practicability. Given that it is simply not possible to measure all biological and chemical parameters of soil systems, we need to rely on so-called proxies defined as: (i) being relevant to the ecosystem under study, (ii) being sensitive enough to report on stressors that would put the system outside of the NOR and (iii) being easy to measure in a range of ecosystems, discriminating between soils that are intrinsically different. These proxies, which will often be microbially-based, should encompass

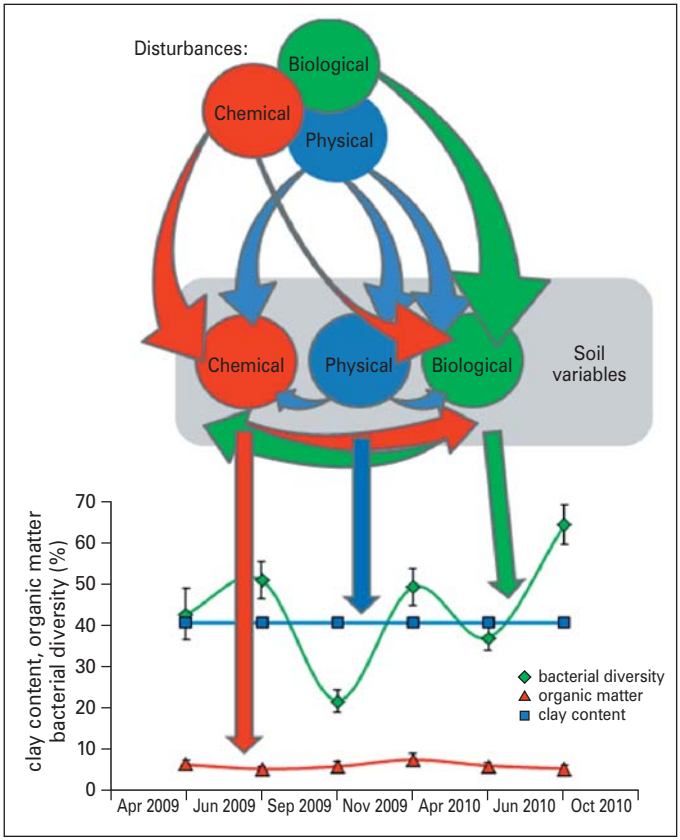

Figure 1.1. Representation of the fluctuations of soil microbial community, and how they are influenced by physical, chemical and biological soil parameters. Moreover, whereas some physical and chemical parameters are quite stable over time, some biological parameters can show great fluctuations.

key ones that are involved in important and potentially sensitive ecosystem processes (e.g. steps of the biogeochemical cycles). They should also represent integrated properties of the environment. However, the question remains: what constitutes a good proxy to serve as a biological indicator, here also called bioindicator? A number of target bioindicators have been suggested (Box 1). They can be based on the structure, size and activity of the soil microbial communities, on diversity measures (functional, taxonomic, genetic), on functions and their contributions to soil processes, on measures for the resilience, resistance, robustness and stability of soil LSF, and/or on the trophic structure related to the soil food web (Winding *et al.*, 2005). In general, bacteria are considered to be important sources to base bioindicators upon, because they numerically

dominate the soil prokaryote communities (van Elsas *et al.*, 2006), are able to function outside of plants, can otherwise function in interaction with plants during the entire life cycle of the latter, and may precisely indicate environmental changes. However, due to the functional redundancy in microbial systems, disturbance-sensitive species can be replaced by stress-tolerant ones, leading to the fact that diversity can be altered without significant effects on function (Nannipieri *et al.*, 2003). For instance, by determining the baseline of bacterial diversity associated with a suite of potato plants, Inceoglu *et al.* (2011) showed that the putative physiological changes in a GM potato versus the near-isogenic parent had no effect on the bacterial community associated with its rhizosphere. In fact, the patterns fell within the established baseline of the potato-related bacterial diversity. Less functionally redundant microbial groups such as those associated with processes like nitrogen fixation and nitrification have been advocated as quite suitable proxies to describe soil "normality", in particular in the context of the assessment of the risk of GM plants (Domsch *et al.*, 1983; Kowalchuk *et al.*, 2003; Bruinsma *et al.*, 2003).

Nitrogen is a key element for many biological molecules (that are involved in cellular processes), like proteins and nucleic acids. It is essential for plant growth, often representing an important constraint to agricultural productivity (Barrios 2007). Biological nitrogen fixation (BNF), the reduction of atmospheric N2 gas to biologically-available ammonium, lies at the basis of all life on Earth. It is performed by phylogenetically diverse groups of prokaryotic microorganisms belonging to the *Bacteria* and the *Archaea* (Young, 1992), which harbor the *nif*H gene, one of the genes coding for the structural part of nitrogenase. When reduced N is a limiting nutrient, BNF can play an important role in providing N inputs to the cropping system budget (Barrios 2007). The diazotrophic organisms occur either as free-living ones or in association with plants (symbiotic or not), and symbiotic nitrogen-fixing bacteria are known to be highly sensitive to perturbation (Doran and Safley, 1997). Several environmental factors have been suggested to influence the N fixation, including soil moisture, oxygen, pH, C quantity and quality, N availability (Hsu and Buckley, 2009), soil texture and aggregate size (Poly *et al.*, 2001b), climate (Mergel *et al.*, 2001) and clay content (Roper and Smith, 1991). The susceptibility of the N-fixing communities to external changes classifies them as potentially important bioindicators of soil health.

Another sensitive step on the nitrogen cycling is ammonia oxidation, which is the first and rate-limiting step in the nitrification process. In this process, ammonia monooxygenase (encoded by the so-called *amo* gene) is the key functional enzyme. There is increasing evidence that ammonia oxidation can be severely impacted by major impacts on the soil system (Kowalchuk and Stephen, 2001). It has long been considered to be performed largely by autotrophic ammonia-oxidizing bacteria (AOB) from two distinct monophyletic

groups within the γ- and β- proteobacteria. However, ammonia oxidizers belonging to the domain archaea (AOA) have been identified about 7 years ago (Könneke *et al.*, 2005; Schleper *et al.*, 2005; Treusch *et al.*, 2005). These were found to dominate in several soils (Leininger *et al.*, 2006), although the exact contribution of each one of these communities to the local nitrification rates remains unclear. AOB have been frequently used as indicators of perturbation, e.g. to measure effects of pollution in fish farm sediments (McCaig *et al.* 1999), contamination of soil with toxic metals (Stephen *et al.*, 1999), effect of effluent irrigation (Oved *et al.*, 2001) and of organic waste residues (Horz *et al.*, 2004; Nyberg *et al.*, 2006). Moreover, due to their presumed niche differentiation and different susceptibility to environmental change, both AOA and AOB have been proposed as indicators of soil disturbance (Wessén and Hallin, 2011).

Box 1: Other pertinent indicators of soil health or quality

Soil quality and soil healthy are important concepts. Soil health has been defined as "the capacity of soil to function as a vital living system to sustain biological productivity, promote environmental quality and maintain plant and animal health" (Doran and Zeiss 2000). Soil health/quality indicators are measurable soil attributes that affect the capacity of soil to support crop production or perform ecosystem functions (Arshad and Martin 2002). These might include biological, chemical and/or physical soil measurements. Potential physico-chemical indicators of soil quality might include pH, cation exchange capacity, changes in organic matter, bulk density, water retention and porosity (Larson and Pierce 1991). Furthermore, soil osmotic and matric potential may be important (Chowdhury ***et al.***, 2011). Soil organic matter is considered to be one of the most important indicators of soil quality due to its association with different soil chemical, physical and biological processes (Silveira ***et al.***, 2009). Biochemical properties of soil, such as microbial biomass, microbial respiration, chitinase and acid-phosphatase activities have been also used as indicators to assess the impact of agricultural management on changes in organic C (Lagomarsino ***et al.***, 2009). Soil fauna is also considered to be important in soil ecosystems (Wolters 2000; Osler and Sommerkorn, 2007); soil quality indices based on microarthropods have been provided (Paolo ***et al.***, 2010; Yan ***et al.***, 2012; Cluzeau ***et al.***, 2012). Although physico-chemical properties of soils have a fundamental utility in soil quality assessments, providing the fundamental context in which functions are performed, the majority of soil processes are driven by the soil microbiota (Ritz ***et al.***, 2009).

Molecular approaches for assessing soil disturbances

A broad range of methods can be used to study the microbial communities in soil. These methods are usually divided into cultivation-dependent and cultivation-independent approaches. Cultivation-dependent methods address those microorganisms that are readily culturable *per se*. However, to unravel the microbial ecology of natural soil systems, the cultivation-based methods are not sufficient, as outlined in the foregoing. Hence, nucleic-acid based techniques have started to dominate the analyses of the soil microbiota made, allowing an assessment of soil microbial community structure and function. The development of molecular methods, for which DNA and RNA needs to be extracted from environmental samples, has started a new era in microbial ecology, as this novel methodology allowed the study of the total microbial communities, including the non-culturable microorganisms. Theoretically, the microbial DNA isolated from a soil sample represents the collective DNA of all the indigenous soil microorganisms. This has been coined the soil metagenome (Handelsman *et al.*, 1998; Rondon *et al.*, 1999).

Because of the complexity of the soil sample, DNA should be properly extracted from soil and purified. This is crucial since the principal source of bias in any molecular soil microbial community analysis lies in the initial extraction of nucleic acids from soil (Frostegard *et al.*, 1999; Griffiths *et al.*, 2004; Lombard *et al.*, 2011). The soil DNA extraction step should be carefully done particularly in organic soils, where co-extracted organic material can interfere with subsequent analysis.

A short description of the molecular methods that were used in this thesis, together with their possible applications, is given below:

1. Denaturing gradient gel electrophoresis (DGGE)

PCR-DGGE and other PCR-based community fingerprinting techniques have revolutionized our understanding of microbial diversity and population dynamics in soil (Sessitsch *et al.*, 2006). The DGGE analysis of PCR-amplified 16S rRNA gene fragments or functional genes came up as an alternative to other time-consuming molecular strategies, e.g. cloning and sequencing. In DGGE, DNA fragments of the same length but with different base-pair sequences can be separated, based on the changed electrophoretic mobility of a partially melted DNA molecule in a polyacrylamide gel containing a linearly increasing gradient of DNA denaturants (urea and formamide) (Muyzer *et al.*, 2004). The number and position of fragments reflect the microorganisms in the community (Winding *et al.*, 2005). PCR-DGGE thus allows the direct visualization of the bacterial community structure via a fingerprint (banding pattern) in a polyacrylamide gel. Such PCR-based fingerprints have a fair resolution and provide information about changes in the structure of the microbial community,

in particular of the dominating types in these (Torsvik and Ovreás, 2002). Among the fingerprinting methods available, the method of choice for this project will be DGGE since it has been applied successfully to monitor microbial communities in soil (Salles et al 2006; Gelsomino et al, 1999; Myuzer and Smalla, 1998). However, as stated, the fingerprints will mainly detect the dominant community members, leaving the rare ones undetected. Such minority populations can, however, be detected by group-specific PCR-DGGE or via quantitative PCR approaches.

In addition to the systems based on the phylogenetic marker, other PCR-DGGE systems that target genes encoding proteins involved in specific metabolic steps have started to be used in microbial ecology as well. For instance, genes such as *amo*A, encoding for ammonia monooxygenase, *nar*G for nitrate reductase, *nif*H for nitrogenase and others have been used to address the nature and diversity of bacterial species involved in different steps of the nitrogen cycle (Francis *et al.*, 2003; Nicol *et al.*, 2008; Rosado *et al.*, 1998; Poly *et al.*, 2001; Pereira e Silva *et al.*, 2011). For instance, Tourna *et al.* (2008) used DGGE based on *amo*A gene determined the influence of temperature on the response of ammonia-oxidizing bacteria (AOB) and archaeal (AOA) in nitrifying soil microcosms. The authors observed changes in community structure of AOA due to incubation in different temperatures, but not in the structure of AOB. DGGE has also been used to assess the genetic diversity of nitrogen-fixers in the rhizospheres of two cultivars of sorghum, based on *nif*H gene (Coelho *et al.*, 2008), where changes in community structure in response to fertilization was observed.

2. Quantitative PCR (qPCR)

Real-time PCR is a highly sensitive tool capable of detecting and quantifying PCR products during a PCR reaction through the production of fluorescence during the amplification reaction. The fluorescent emission from one of the dyes, the reporter, is quenched by the emission from the other dye (Hermansson and Lindgren, 2001), and progressively liberated during amplification. Polymerase chain reaction (PCR), as explained in 2.3.1., has primarily been used as a qualitative method to confirm the presence of a specific DNA sequence in a sample (Jansson and Leser, 2004). Quantification of DNA by real-time PCR is based (using an external standard curve) on measurements obtained during the early exponential phase of amplification, the only phase where the amount of amplified target is directly proportional to the initial amount of target molecules.

With q-PCR, several bacterial groups have been quantified in environmental samples, e.g. the phytopathogenic bacterium from citrus, *Xyllela fastidiosa* (Oliveira, 2002), as well as non-pathogenic bacteria like the endophytic colonizer of *Catharanthus roseus*, *Methylobacterium mesophilicum* (Lacava *et al.*, 2006). Also, the approach has been used to quantify functional genes, e.g. *amo*A

(Okano *et al.*, 2004; Hermansson and Lindgren, 2001). In fact, q-PCR can be used to quantify virtually any target gene. Wallenstein and Vilgalys (2005), for example, developed a qPCR technique to quantify several nitrogen cycling genes (*amo*A, *nif*H, *nir*S, *nir*K, *nos*Z) by quantitative real-time PCR using SybrGreen as the dye. Using qPCR it was found for instance that AOAs are more abundant in soil than AOBs (Leininger *et al.*, 2006), and that abundance of *nif*H gene was lower in some strongly acidic surface soil sites in North-East Victoria (Dermosols), while *nif*H was more abundant in selected Calcarosols of North-West Victoria in Australia (Hayden *et al.*, 2010).

3. Pyrosequencing

Massive parallel pyrosequencing systems have increased the efficiency of DNA sequencing and also the resolution of the microbial community structures. These techniques have emerged given the fact that only a superficial picture of the microbial community structure of a soil can be obtained with clone libraries (Dunbar *et al.*, 2002). The 16S rRNA gene, next to functional gene based amplicon pyrosequencing, permits a much deeper sampling of microbial communities by providing orders of magnitude more sequence information than traditional Sanger sequencing of PCR-generated clone libraries (Engelbrektson *et al.*, 2010). Pyrosequencing has been applied to investigate the fluctuations in microbial communities over time, and have revealed that bacterial community and also functional community composition varies among and within lakes (Hutalle-Schmelzer and Grossart, 2009; Jones *et al.*, 2009) and soils (Mao *et al.*, 2011; Palmer *et al.*, 2012) over temporal and spatial scales.

The normal operating range (NOR) of soils – the search for a reference system

The concept of NOR date from the seventies, when Odum *et al.* (1979) introduced the idea in their ecosystem perturbation theory. They stated that perturbation is any deviation or displacement from the nominal state, which encompasses a range of fluctuating conditions under which functioning is normal. Although there is an urge for a NOR of soil functioning to be developed, up to now it has not been very well defined. Briefly, the NOR can be represented by the natural fluctuations of soil health/quality indicators; it thus determines the 'natural' limits of variation in soil functioning. According to the nature and intensity of the disturbances, higher or lower variations in soil processes are expected. In practical terms, the NOR offers a statistical tool that provides a "normal" score for soil functioning, which would likely be location-, soil type and/or management- dependent. The relevance of soil type when defining the NOR has been defined as a key factor in studies focusing on macroorganisms,

where the gene expression of the soil-dwelling collembolan *Folsomia candida* was differentially regulated in clay versus sandy soil (de Boer *et al.*, 2011).

The NOR will thus provide a background against which to compare soil conditions incited by, for instance, genetically-modified (GM) crops or other factors, and forms the reference or baseline against which the effects of external disturbances (e.g. anthropogenic emissions) can be judged (Meier *et al.*, 2008; Kowalchuk *et al.*, 2003; Bruinsma *et al.*, 2003), providing a key monitoring tool for policy makers.

Aim of this thesis and research questions

The major purpose of this thesis is to investigate natural fluctuation in key soil microbial-related processes across a range of selected Dutch soils, in order to yield a tool useful for description of the NOR. This may support regulatory agencies in ecological assessments in their task to evaluate the effects of disturbances on soil systems.

In this study, eight representative soils across the Netherlands were selected: Buinen (B), Valthermond (V), Droevendaal (D), Wildekamp (W), Kollumerwaard (K), Steenharst (S), Grebbedijk (G) and Lelystad (L). The field soils were found to have different characteristics in terms of pH, texture, organic matter content and nitrate and ammonium levels All fields were used for potato cropping and subjected to (1:4) crop rotation with non-leguminous plants, except soil W, which contained a permanent grassland (Table 1.1; See also Table 3.1 for a more detailed description). Taking into account data from a 3-year sampling period, we analyzed the abundances and community structures of *Archaea*, *Bacteria* and *Fungi*, as well as ammonia oxidizers and nitrogen-fixers. Moreover, we measured key soil chemical parameters and potential activities. The final aim was to build a large dataset suitable for assessments of soil functioning in agricultural

Table 1.1. Soils collected in this thesis and crops present in each field.

Soils	2008	2009	2010	2011
Buinen (B)	Potato	Barley	Potato	Potato
Valthermond (V)	Barley	Barley	Potato	Potato
Droevendaal (D)	Oat	Triticale	Barley	Barley
Wildekamp (W)	Grass	Grass	Grass	Grass
Steenharst (S)	Potato	Potato	Grass	Sugar beet
Kollumerwaard (K)	Potato	Grass	Potato	Grass
Grebbedijk (G)	Wheat	Potato	Wheat	Wheat
Lelystad (L)	Carrots	Potato	Grass	Corn

soils, and to provide a tool to establish the NOR of soil functioning, allowing the detection of soils undergoing disturbances. As an overriding hypothesis underlying this work, I posit that all soils under agricultural use show fluctuations in their function, as a result of normal climatic factors next to normal agricultural regime. The challenge of the study described in this thesis was, thus, to disentangle normality – which establishes a range within which functioning can be considered as typical, or within the range, for a given soil - from abnormality.

In the work described in this thesis I aimed to answer the following research questions:

- To what extent do the aforementioned soil communities fluctuate with respect to community composition, abundance, structure and function?
- To what extent are these communities influenced by soil parameters, in particular by pH and texture?
- To what extent can particular functional groups, representing key microbial processes, be used as proxies of environmental disturbances?
- How can the NOR of soil functioning be established, taking into account the natural dynamics of soil processes and communities?

Outline of the thesis

These above research questions stated above have been addressed in from chapters 2 to 9.

In **chapter 2** I explore the temporal and spatial responses of soil bacterial, archaeal and fungal communities to abiotic parameters, taking into account data from a 3-year sampling period and all soil sites. The main idea was to examine the responsiveness of these groups to soil parameters, and their potential usage as indicators of soil disturbances. From this chapter, it became clear that archaea were more sensitive responders than bacteria and fungi. The latter groups didn't significantly respond to important soil parameters measured.

The results obtained in the previous chapter supported the idea that the functional redundancy of bacterial, fungal, and to a lesser extent, archaeal communities would hinder their use as sensitive bioindicators. Thus in **chapters 3** and **4** I describe the dynamics of two microbial functional groups, which are expected to be more susceptible to fluctuations due to their lower redundancy. **Chapter 3** describes the dynamics in the abundance and structure of diazotrophic communities, based on the *nif*H gene, during one complete growing season. This study was performed to evaluate the amplitude of the natural variation in abundance and diversity, and to identify possible relationships with abiotic factors. **Chapter 4** addresses the effects of soil abiotic parameters on the abundance, structure and function of the soil ammonia oxidizers. For this, the eight agricultural soils were collected and analyzed across the Netherlands over two years.

This study describes the baseline for nitrification and its proxies. The data might be used as a basis when defining the NOR of nitrification in agricultural soils.

The responsiveness of nitrogen fixers and ammonia oxidizers to biotic and abiotic parameters encouraged a more thorough description of the natural variations in community composition (structure and diversity), attempting the identification of suitable indicators of "normal" soil status. **Chapter 5** provides an evaluation of the temporal and spatial changes in the soil diazotrophic composition. The study was based on the *nif*H gene, and employed a deep-sequencing strategy and analysis. **Chapter 6** provides a similar analysis based on the *amo*A gene. As I observed high correlations between the AOA community, soil characteristics and nitrification rates in **Chapter 4**, in **Chapter 6** I focus on archaeal ammonia oxidizers (AOA). This was done to test the hypothesis that AOA represent a sensitive functional group and to evaluate the extent to which abiotic parameters are related to changes in AOA composition across temporal and spatial scales.

From the previous results, it was clear that soil pH and texture were the major drivers determining changes in microbial community abundance, structure and activity. However, as these two factors co-vary in this study (clay soils had higher pH whereas sandy soils were more acidic), in **Chapter 7** I set up microcosm experiments, as these were deemed to validate particular groups and their responsiveness to soil factors. More specifically, this chapter describes the influence of soil texture and soil pH on the abundance and function of nitrogen fixers and ammonia oxidizers. Moreover, I aimed to identify the main responders, which would constitute the best indicators.

In **Chapter 8** and **9** I address how the information acquired in the previous chapters could be used to define the normal operating range of soils. **Chapter 8** discusses the pertinent and important literature concerning the development of the NOR of soil, as well as the main caveats inherent to the concept. A special focus was given to nitrification as it has been advocated that it can provide suitable proxies to describe soil status and "normality" (Domsch *et al.*, 1983; Kowalchuk *et al.*, 2003; Bruinsma *et al.*, 2003). Our results reaffirms that proxies related to nitrification are important, as they describe a sensitive indicator group. Such proxies, next to other facets of soil, are key components of the model that describes the normal operating range of soil. This model is presented in **Chapter 9**, which describes an approach based on the fluctuations of several physical, chemical and biological indicators, allowing the visualization in a multidimensional space of "stressed" situations. Thus, prototype-monitoring tool for judgment of soil normality was proposed.

In **Chapter 10** I synthesize the various insights gained from this thesis and discuss the overall results obtained. In particular, I focused on how the fluctuations in soil microbial community and function can be integrated in the NOR, and used as a tool to detect soil systems under disturbance.

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Chapter 2

Spatial and temporal variation of archaeal, bacterial and fungal communities in agricultural soils

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Abstract

Background: Soil microbial communities are in constant change at many different temporal and spatial scales. However, the importance of these changes to the turnover of the soil microbial communities has been rarely studied simultaneously in space and time.

Methodology/Principal findings: In this study, we explored the temporal and spatial responses of soil bacterial, archaeal and fungal β-diversities to abiotic parameters. Taking into account data from a 3-year sampling period, analyzing the abundances and community structures of *Archaea*, *Bacteria* and *Fungi* and key soil chemical parameters, we questioned how these abiotic variables influence the turnover of bacterial, archaeal and fungal communities. Moreover, we investigated how they impact the long-term patterns of changes of the aforementioned soil communities. Interestingly, we found that the bacterial and fungal β-diversities are quite stable over time, whereas the archaeal one showed significantly higher fluctuations, which were reflected in temporal turnover caused by soil management through addition of N-fertilizers.

Conclusions: Our study showed that management practices applied to agricultural soils might not significantly affect the bacterial and fungal communities, but cause slow and long-term changes in the abundance and structure of the archaeal community. Moreover, the results suggest that abiotic and biotic factors determine the community assembly of archaeal, bacterial and fungal communities to different extents.

Introduction

Understanding temporal and spatial patterns in the abundance and distribution of communities has been a fundamental quest in ecology. Such an understanding is crucial to allow an anticipation of responses of ecosystems such as soil to global changes (Singh *et al.*, 2010). Because local conditions are never constant, small disturbances that affect the soil microbial communities might occur [Hooper and Vitousek, 1997; Tilman *et al.*, 1997) at different temporal and spatial scales. The assessment of microbial communities at a particular locality may result in patterns that vary greatly both within and between years, and these communities may be subjected to changes over longer time scales as a result of processes such as succession and evolutionary change (Bardgett *et al.*, 2005). One approach to investigate temporal (and spatial) variability in complex systems is to explore patterns of β-diversity. Whereas alpha (α-) diversity represents a measure of the total diversity of a given site, β-diversity is the variation of species composition (turnover) across space or time between paired sites. High β-diversity indicates large differences in community composition among different sites. Such high diversity can result from local as well as regional factors, e.g. changes in the local environmental conditions or limitation of dispersal between them (Lindström and Langenheder, 2011).

Temporal variation of conditions is a very common feature of ecosystems. Ecologists have long been interested in how such variation structures natural communities (Andrewartha and Birch, 1953; Lewontin and Cohen, 1969). It can presumably affect the rate of microbial turnover, as microorganisms can process resources and adapt to changes in natural environments on a much faster time scale than macroorganisms (Schmidt *et al.*, 2007). Moreover, many functional microbial groups can show dramatic seasonal changes in soils (Lipson *et al.*, 2002).

The number of studies employing the concept of β-diversity to understand how microbial communities respond to biotic and abiotic parameters has increased substantially in soil ecology. Martiny and co-workers (Martiny *et al.*, 2011) studied the mechanisms driving ammonia-oxidizing bacterial (AOB) communities in salt marsh sediments. They found no evolutionary diversification when comparing the AOB community composition between three continents; although a negative relationship was observed between geographic distance and community similarity. Furthermore, in an attempt to determine to which extent a bacterial metacommunity, consisting of 17 rock pools, were structured by different assembly mechanisms (Langenheder *et al.*, 2012), the authors studied changes in β-diversity across different environmental gradients over time, including phosphorus concentration, temperature and salinity. They found that there were temporal differences in how the communities respond to abiotic factors. β-diversity allows not only the understanding of temporal but of

spatial variations as well. For instance, in a survey of bacterial communities across more than 1000 soil cores in Great Britain (Griffiths *et al.*, 2011), no spatial patterns were observed, but instead variations in β-diversity according to soil pH were found, which revealed that β-diversity (between sample variance in α-diversity) was higher in acidic soils (pH 4-5) than in more alkaline soils (pH 7-9) (Griffiths *et al.*, 2011). In the former soils, environmental heterogeneity was highest, calculated as the variance in environmental conditions (Griffiths *et al.*, 2011). In another study, different patterns of bacterial β-diversity were observed between different layers in sediment cores, which could be attributed to historical variation and geochemical stratification (Wang *et al.*, 2008).

Of the soil microbial groups, bacteria have been mostly studied, as they exhibit an estimated species diversity of about 10^3 to up to 10^6 per g soil Curtis *et al.*, 2002; Gans *et al.*, 2005; Torvisk *et al.*, 2002). However, archaea and fungi are also important microorganisms found in soil. Previous studies have shown the ubiquity of archaea in soil, especially the crenarchaeota (Buckley *et al.*, 1998; Jurgens *et al.*, 1997; Ueda *et al.*, 1995). Fungal abundances in the order of 10^4 fungal propagules per g of dry soil were observed in Antarctic soils (Jung *et al.*, 2011) and 10^7 per g of soil in soil crusts (Bates *et al.*, 2011). Fundamental differences in the physiology and ecology of members of such communities would suggest that their patterns of spatial and temporal variation are controlled by distinct edaphic factors.

In this study, we explored the temporal and spatial fluctuations of soil microbial communities and their relation to local environmental conditions. In order to do so, we investigated the spatiotemporal dynamics of the soil microbiota by analyzing the patterns of α- and β-diversity of archaea, bacteria and fungi in eight agricultural soils across the Netherlands. We sampled the soils eleven times, from 2009 to 2011. Furthermore, to complement the analyses, we applied TLA (time-lag analysis) (Collins *et al.*, 2000), a distance-based approach to study the temporal dynamics of communities by measuring community dissimilarity over increasing time lags. TLA provides measures of model fit and statistical significance, allowing the quantification of the strength of temporal community change in a numerical framework (Angeler *et al.*, 2009). We thus interrogated how the relationship between microbial abundance, species composition and the surrounding environment varies in space and time, and how this relates to long-term compositional changes.

Material and Methods

Study area and field sampling

The eight soil sites sampled are located in the Netherlands. Their characteristics and geographical coordinates are found in Table 2.1 and in Table S1. Sampling

points were selected to reflect temporal differences in external parameters. For each soil four replicates were taken. Each replicate consisted of 10 subsamples (15–20 cm deep) collected between plots, away the roots with a spade. Soil samples were collected four times over an annual cycle in 2009 (April, June, September and November), three times in 2010 (April, June and October), and four times in 2011 (February, April, July and September). Each sample was placed in a plastic bag and thoroughly homogenized before analysis. A 100-g subsample was kept at 4°C and used for chemical analyses, whereas the remaining soil was kept at –20°C for subsequent DNA extraction and molecular analysis of bacterial, archaeal and fungal community compositions and total abundance (see below).

Soil chemical analysis

The environmental variables measured included pH, concentrations of nitrate ($N\text{-}NO_3^-$ in mg/kg of soil), ammonium ($N\text{-}NH_4^+$ in mg/kg of soil), organic matter (OM in %) and clay content (in %). The pH was measured in CaCl2 suspension 1:4.5 (g/v) (Hanna Instruments BV, IJsselstein, The Netherlands). Organic matter (OM) content is calculated after 4 hours at 550°C. Nitrate ($N\text{-}NO_3^-$) and ammonium ($N\text{-}NH_4^+$) were determined with a colorimetric method using the commercial kits Nanocolor Nitrat50 (detection limit, 0.3 mg N kg^{-1} dry weight, Macherey-Nagel, Germany) and Ammonium3 (detection limit, 0.04 mg N kg^{-1} dry weight; Macherey-Nagel, Germany) according to Töwe *et al.* (2010).

Table 2.1. List of soils included in this study.

Sampling Site	Soil type	Land use	Crops			North coordinate	East coordinate
			2009	2010	2011		
Buinen (B)	Sandy loam	agriculture	barley	potato	potato	52°55′386″	006°49′217″
Valthermond (V)	Sandy loam	agriculture	barley	potato	potato	52°50′535″	006°55′239″
Droevendaal (D)	Sandy loam	agriculture	triticale	barley	barley	51°59′551″	005°39′608″
Wildekamp (K)	Sandy loam	grassland	grass	grass	grass	51°59′771″	005°40′157″
Kollumerwaard (K)	Clayey	agriculture	potato	grass	sugar beet	53°19′507″	006°16′351″
Steenharst (S)	Silt loam	agriculture	grass	potato	grass	53°15′428″	006°10′189″
Grebedijk (G)	Clayey	agriculture	potato	wheat	wheat	51°57′349″	005°38′086″
Lelystad (L)	Clayey	agriculture	potato	grass	corn	52°32′349″	005°33′601″

Nucleic acid extraction

DNA was extracted from 0.5g of soil using Power Soil MoBio kit (Mo Bio Laboratories Inc., NY), according to the manufacturer's instructions, after the addition of glass beads (diameter 0.1 mm; 0.25 g) to the soil slurries. The cells were disrupted by bead beating (mini-bead beater; BioSpec Products, United States) three times for 60 s. Following extraction, the DNA preparations were electrophoresed over agarose gels in order to assess DNA purity, quality (average size) and quantity. The quantity of extracted DNA was estimated on gel by comparison to a 1-kb DNA ladder (Promega, Leiden, Netherlands) and quality was determined based on the degree of DNA shearing (average molecular size) as well as the amounts of coextracted compounds.

Real-time PCR quantification (qPCR)

Absolute quantification was carried out in four replicates on the ABI Prism 7300 Cycler (Applied Biosystems, Germany). The 16S rRNA gene was amplified by qPCR using diluted extracted DNA as template and specific primers for archaea (group 1 crenarchaeota) 771F/ 957R (Ochsenreiter *et al.*, 2003) and for V5-V6 region of bacteria 16SFP/ 16SRP (Bach *et al.*, 2002) were used. We have chosen to focus on this group as Crenarchaeota is often more common in soil environments than Euryarchaeota (Nicol *et al.*, 2004). For Fungi communities primers 5,8S/ ITS1f (Fierer *et al.*, 2005) were chosen. Cycling programs and primer sequences are detailed in Table S2. The specificity of the amplification products was confirmed by melting-curve analysis and on 1.5% agarose gels. Standard curves were obtained using serial dilutions of plasmid containing the cloned 16S rRNA gene obtained from *Burkholderia terrae* BS001 or ITS region of *Rhizoctonia solani* AG3. Dilutions ranged from 10^7 to 10^2 gene copy numbers/µl. Archaeal standard curve was obtained by serial dilution of PCR product generated from *Cenarchaeum symbiosum* with the aforementioned archaeal specific primers (Ochsenreiter *et al.*, 2003).

PCR for DGGE analysis

For DGGE analysis, bacterial 16S rRNA genes were PCR amplified using the forward primer F968 [28] with a GC-clamp attached to 5′ and the universal R1401.1b (Brons and van Elsas, 2008). Archaeal 16S rRNA genes were amplified with the A2F/U1406R primer pair (Bano *et al.*, 2004), following amplification using the *Archaea*-specific forward primer at position 344 with a 40-bp GC clamp (Myers *et al.*, 1985) added to the 5′ end, and a universal reverse primer at position 517. The fungal ITS region was amplified with EF4 (Smit *et al.*, 1999)/ITS4 (White *et al.*, 1990), followed by a second amplification with primers ITS1f-GC (Gardes and Bruns, 1993) /ITS2 (White *et al.*, 1990). PCR mixtures, primer sequences and cycling conditions are described in Table S3. About 200ng of amplicons were loaded on onto 6% (w/v) polyacrylamide gels in the Ingeny

Phor-U system (Ingeny International, Goes, The Netherlands), with a 20–50% denaturant gradient for fungi community, 45–65% for bacterial and 40–60% for archaeal community (100% denaturant corresponded to 7 M urea and 40% (v/v) deionized formamide). Electrophoresis was performed at a constant voltage of 100 V for 16 h at 60ºC. The gels were stained for 60 min in 0,5x TAE buffer with SYBR Gold (final concentration 0,5 µg/liter; Invitrogen, Breda, The Netherlands). Images of the gels were obtained with Imagemaster VDS (Amersham Biosciences, Buckinghamshire, United Kingdom). Genetic fingerprints were analyzed using GelCompar software (Applied Maths, Sint-Martens Latem, Belgium) (Kroft, 2004; Rademaker and Bruijn, 1999).

Data analyses

The diversity of each of the soil bacterial, archaeal and fungal communities was determined on the basis of the PCR-DGGE profiles. Total diversity (α) of the dominant community members was estimated from these data using the Shannon index, as recommended by Hill *et al.* (2003), as well as the number of DGGE bands (species richness). We calculated the temporal β-diversity of archaeal, bacterial and fungal communities as the mean of all pairwise Bray-Curtis dissimilarities based on the relative abundance of DGGE bands, as previously described (Legendre *et al.*, 2005; Peres-Neto *et al.*, 2006; Langenheder *et al.*, 2012). To support result from the calculated β-diversity and to test he statistical significance and the strength of community dynamics we used time-lag analysis (TLA) (Collins *et al.*, 2000) by plotting Hellinger-transformed (Legendre and Gallangher, 2001) distance values against the square root of the time lag for all lags. The time-lag analytical approach can produce a number of general theoretical patterns with time-series data (Collins *et al.*, 2000). The square root transformation reduces the probability that smaller number of points at larger time lags will bias the analysis (Kampichler and Geissen, 2005). The Bray-Curtis matrices as well as Hellinger-transformed distances were determined in PRIMER-E (version 6, PRIMER-E Ltd, Plymouth, UK; Clarke and Gorley 2006).

To test how α-diversity, β-diversity and microbial abundance varied in relation to environmental variables, parametric Pearson correlation coefficients were calculated between α and β diversities and soil pH, organic matter, nitrate, ammonium, clay content and soil moisture, as well as between total abundances and TLA slopes using SPSS v18.0.3 (SPSS Inc., Chicago, IL, USA). All variables except pH were transformed (Log(x+1)) prior to all analyses. Moreover, we applied variance partitioning to evaluate the relative contribution of the drivers of the microbial assemblages. Forward selection was used on CCA (Canonical Correspondence Analysis), to select a combination of environmental variables that explained most of the variation observed in the species matrices. For that, a series of constrained CCA permutations was performed in Canoco (version 4.0 for Windows, PRI Wageningen, The Netherlands,) to deter-

mine which variables best explained the assemblage variation, using automatic forward selection and Monte Carlo permutation tests (permutations = 999). The length of the corresponding arrows indicated the relative importance of the chemical factor explaining variation in the microbial communities.

Results

Variability of environmental parameters

Soil pH, nitrate, ammonium and organic matter levels were determined in triplicate across all soil samples. Soil pH was significantly higher ($P < 0.05$) in soils K, G and L (7.32 ± 0.06, n = 57) than in soils B, V, D, W and S (4.88 ± 0.04, n = 99) during the whole experimental period and no significant variation over time was observed. In all soils, significant changes were observed in the levels of nitrate, with lower values at the end of the growing season for most of the soils (September 2009: 32.78 mg/kg ± 7.77; October 2010: 24.15 mg/kg ± 3.62; September 2011: 2.45 mg/kg ± 0.41) and higher at the beginning (April 2009: 75.6 mg/kg ± 12.5; April 2010: 56.4 mg/kg ± 5.63; April 2011:100.1 mg/kg ± 16.5). Levels of ammonium also varied over the whole period, with higher values being observed at the beginning of the season (April 2009: 13.3 mg/kg ± 1.14; April 2010: 16.0 mg/kg ± 1.19; April 2011: 12.1 mg/kg ± 2.72), and lower values at the end (September 2009: 1.93 mg/kg ± 0.16; October 2010: 8.86 mg/kg ± 1.22) (Table S1)

Considering each soil individually, they had characteristically different values, with higher levels of nitrate and ammonium found in soils B, V, D and S than in soils W, K, G and L (Table S1). In 2009 and 2010, variations in organic matter (OM) content were observed from September (5.63% ± 1.20) to November (7.34% ± 1.45) 2009, and from April (6.28% ± 0.85) to June (5.04% ± 0.89) 2010. Small but insignificant variations in OM were observed in 2011. On average, the OM content of all soils was in the range around 4%, except for soil V, which had on average 17% OM.

Temporal variations in the abundance of archaeal, bacterial and fungal communities and their responses to abiotic variables

We studied the variations in the abundances of archaeal, bacterial and fungal communities over time, across all samples in three years. The total bacterial abundance showed significant temporal variation during the whole period, ranging between 8.12 ± 0.23 (mean ± standard error) (September 2011) and 10.93 ± 0.06 (June 2010) log copy numbers per g dry soil and showing comparable copy numbers in sandy (9.65 ± 0.13) and clayey soils (9.64 ± 0.16). The archaeal abundance (crenarchaeota) ranged between 6.96 ± 0.14 (April 2009) and 8.78 ± 0.07 (April 2011) log copy numbers per g dry soil, and showed significant dif-

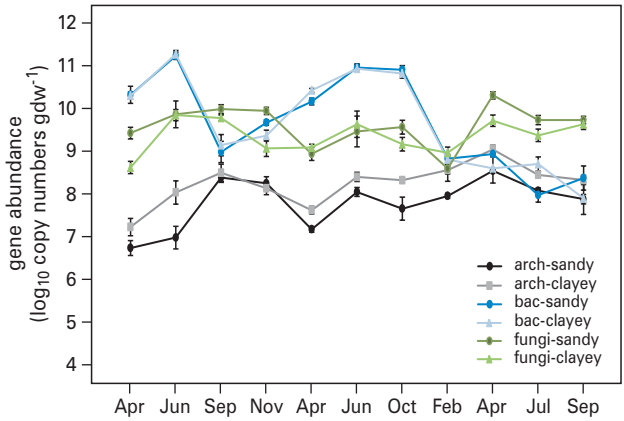

Figure 2.1. Changes in abundance of archaeal, bacterial and fungal communities. The copy number in each gram of dry soil was estimated by real-time PCR in the eight agricultural soils as an average of sandy and clayey soils at different sampling times. Bars are standard errors (n = 4).

ferences between sandy and clayey soils across almost all sampling times, with lower numbers in the sandy soils (7.77 ± 0.13) than in the clayey soils (8.22 ± 0.13). Fungal abundance varied between 8.76 ± 0.16 (February 2011) and 10.00 ± 0.09 (April 2011), and significantly higher abundance was observed in the sandy soils depending on the sampling time (Fig. 2.1). Overall and on average, the abundance of bacteria was higher than that of the fungi, except in September 2009, and during 2011.

We used Pearson's correlation to examine how soil parameters influenced the abundances of bacterial, archaeal and fungal communities. Whereas the archaeal abundances were positively correlated with soil pH ($r = +0.883$, $P < 0.001$), they were negatively influenced by nitrate ($r = -0.764$, $P < 0.05$). A positive relationship was observed between fungal abundance and soil organic matter ($r = +0.722$, $P < 0.05$), and a negative one between fungal abundance and archaeal abundance ($r = -0.484$, $P < 0.05$). Relationships between the abundance of bacteria and fungi, and bacteria and archaea were not significant. Interestingly, none of the soil parameters measured influenced bacterial abundance significantly.

Patterns of α-diversity and response to abiotic variables

Understanding how species are distributed in space and time may yield a first avenue towards their assembly rules (Magurran and Dornelas 2010). We used two ecological measures, i.e. the Shannon index (H') and species richness, as proxies to study the variations in the α-diversities of the archaeal, bacterial and

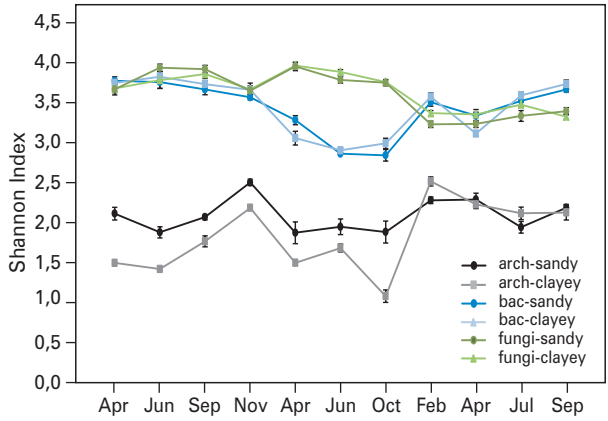

Figure 2.2. Total α-diversity of archaeal, bacterial and fungal communities. Alpha diversity was calculated as the average of the Shannon Index (H') per soil type (sandy x clayey), from April 2009 to September 2011 (mean ± s.d.).

fungal communities. Differential patterns of archaeal, bacterial and fungal α-diversities were observed, as measured by H' (Fig. 2.2). The H' values of the archaeal communities ranged from 1.68 ± 0.04 in June 2009 to 2.40 ± 0.05 in February 2011, and it was consistently lower than the corresponding bacterial and fungal values. The bacterial H' values varied from 2.52 ± 0.04 in October 2010 to 3.85 ± 0.04 in April 2009, whereas those of the fungal communities varied from 3.2 ± 0.16 in April 2009 to 4.09 ± 0.04 in April 2010 (Fig. 2.2). In general, the differences observed between sandy and clayey soils for the bacterial and fungal diversities (Shannon index) were time point dependent. For archaea, a higher Shannon index was noticed in the sandy soils compared to the clayey ones in 2009 and 2010, but not in 2011.

Concerning correlations with edaphic factors, a positive effect of OM content was observed on the archaeal α-diversity ($r = +0.691$, $P < 0.05$) (Table 2.2). When using the number of DGGE bands as a measure of α-diversity (species richness), a significant and strong positive correlation was found between archaeal α-diversity and nitrate levels ($r = +0.962$, $P < 0.001$) (Table 2.2). None of the soil parameters measured correlated significantly with bacterial or fungal α-diversity.

Patterns of temporal β-diversity and responses to abiotic variables

The patterns of temporal β-diversity of the archaeal, bacterial and fungal communities (taking into account the variations in community composition of each microbial group in individual soils over time) showed small but significant variations across soils (Fig. 2.3A). Bacterial β-diversities were in general higher than fungal ones across soils, except for soil V. There were slight but significant

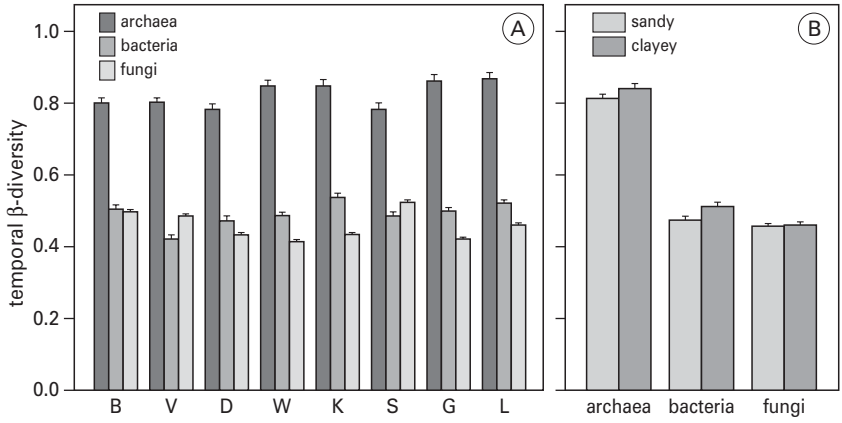

Figure 2.3. Temporal β-diversity of archaeal, bacterial and fungal communities. Temporal β-diversity, which takes into account temporal changes of each individual soil was calculated across the different sampling points (A) and separated per soil type (B) (mean ± s.e.).

Table 2.2. Pearson's correlation coefficient between soil chemical parameters, biotic parameters (total abundance, alpha diversity, beta diversity and slopes from TLA analysis), calculated from the eight soils over time.

	pH ($mg^{-1}kg$)	N-NH_4^+ ($mg^{-1}kg$)	N-NO_3^- (%)	OM (%)	Clay
Total abundance					
Total archaeal community	0.883***	-0.498ns	-0.764*	0.030ns	-0.795*
Total bacterial community	-0.636ns	0.379ns	0.236ns	-0.624ns	0.417ns
Fungi	0.363ns	-0.476ns	-0.356ns	-0.722*	0.387 ns
Alpha Diversity (Shannon)					
Total archaeal community	0.284ns	0.230 ns	0.137ns	0.691*	0.599 ns
Total bacterial community	-0.174ns	-0.033ns	0.470ns	-0.158ns	0.442ns
Fungi	0.095ns	-0.149ns	0.175ns	-0.370ns	0.241ns
Alpha Diversity (N° bands)					
Total archaeal community	-0.408ns	-0.056ns	0.962***	0.482ns	0.442ns
Total bacterial community	0.441ns	-0.355ns	-0.485ns	-0.497ns	-0.335ns
Fungi	-0.154ns	0.150ns	-0.579ns	-0.416ns	-0.469ns
Temporal Beta diversity					
Total archaeal community	-0.194ns	-0.249ns	0.874*	0.541ns	-0.415ns
Total bacterial community	0.028ns	-0.313ns	-0.123ns	-0.502ns	0.074ns
Fungi	-0.380ns	0.167ns	-0.456ns	-0.232ns	-0.035ns

Notes: Values in boldface type indicate significant correlations with P values indicated in superscript.
*P< 0.05; **P< 0.01; ***P< 0.001. ns not significant at P < 0.05.

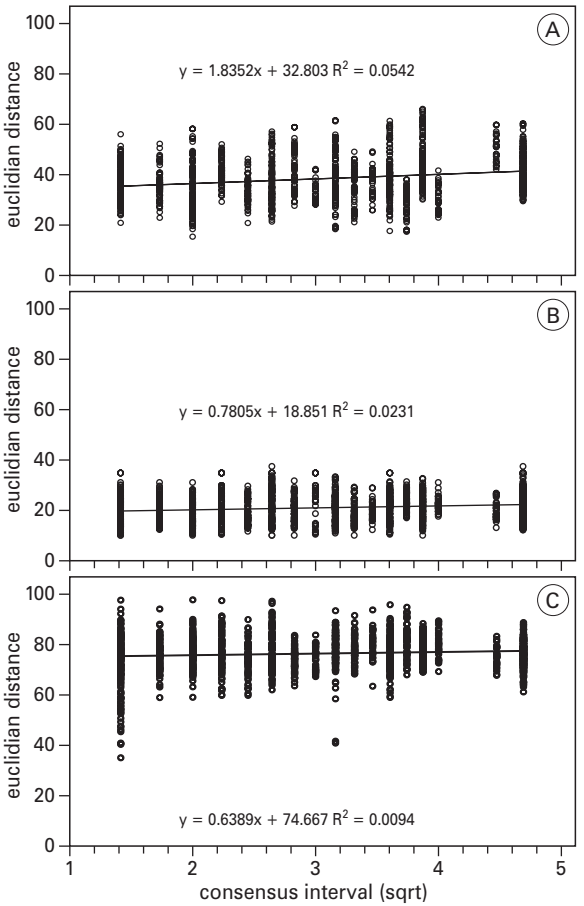

Figure 2.4. Quantification of archaeal, bacterial and fungal dynamics. Patterns of change (regression of square root of time-lag on Euclidian distance) of archaeal (A, slope 1.835), bacterial (B, slope 0.785) and fungal community (C, slope 0.638) in eight soils. The best-fit line is shown.

differences ($P < 0.05$) between sandy and clayey soils regarding the temporal β-diversity of archaeal and bacterial but not of fungal communities (Fig. 2.3B).

Although chemical parameters might show variability over time, still significant correlations could be observed. The patterns in the archaeal temporal β-diversities observed were mainly due to positive correlations with nitrate ($r = +0.874$, $P < 0.05$) (Table 2.2). None of the soil parameters measured was correlated with bacterial and fungal temporal β-diversities. Canonical correspondence analysis was used to test the significance of the influence of soil parameters on the community parameters. We used variance partitioning to control for the

effect of each individual parameter, while all others are defined as covariables in the constrained analyses (Leps and Smilauer 2003). Considering the whole data set, soil parameters explained 45%, 6.6% and 6.9% of the temporal variability in archaeal, bacterial and fungal community structures, respectively. The archaeal community was mostly affected by changes in OM (11.9%) and nitrogen specimens (nitrate + ammonium; 7.8% each), whereas the bacterial and fungal community variations were mostly related to ammonium (2.1% and 2.2% for bacteria and fungi, respectively) (Fig. S1).

Significant relationships were observed between variation in β-diversity and H' for bacterial ($r = +0.602$, $P < 0.05$) and fungal communities ($r = -0.481$, $P < 0.05$), but not for archaeal ones.

Quantifying temporal changes of archaeal, bacterial and fungal communities

The temporal changes of the microbial guilds were quantified and statistically tested via TLA. TLA analyses were performed separately per year and also considering all three years. For both analyses, the results and conclusions were similar. Therefore, we decided to include only the overall data, that is, considering all three years. A statistically significant regression line ($P < 0.05$) was observed for the archaeal community, with an overall slope of 1.835 (Fig. 2.4A and Table 2.3). Moreover, all eight soils showed indications of directional changes in community composition, yielding regression lines that were statistically different from zero ($P < 0.05$) with the exception of the G soil (Table 2.3). Although the slopes were small (Table 2.3), they were mainly reflected in the positive Pearson correlations with nitrate levels ($r = +0.814$, $P < 0.05$). The bacterial communities

Table 2.3. Results of the time-lag analyses (TLA) performed for bacterial, archaeal and fungal communities for all soils separately, and a overall result considering all soils.

Sampling Site	Archaeal community			Bacterial community			Fungal community		
	Slope	P	R^2	Slope	P	R^2	Slope	P	R^2
Buinen (B)	1.749	0.000	0.116	3.298	0.000	0.227	0.989	0.007	0.031
Valthermond (V)	1.876	0.000	0.064	1.903	0.000	0.139	1.297	0.001	0.059
Droevendaal (D)	2.063	0.000	0.098	1.864	0.000	0.135	0.671	NS	0.013
Wildekamp (W)	1.765	0.000	0.105	1.059	NS	0.036	0.414	NS	0.004
Kollumerwaard (K)	1.378	0.028	0.021	1.860	0.009	0.079	-0.078	NS	0.000
Steenharst (S)	2.038	0.000	0.080	1.697	0.015	0.069	0.817	NS	0.012
Grebedijk (G)	0.853	NS	0.022	2.300	0.000	0.156	-0.029	NS	0.000
Lelystad (L)	1.282	0.016	0.026	0.552	NS	0.009	0.981	0.011	0.034
Overall	**1.835**	**0.000**	**0.054**	**0.785**	**0.000**	**0.023**	**0.638**	**0.000**	**0.009**

showed a similar trend as observed for the archaeal ones, with significant regression lines and a slope of 0.785 (Fig. 2.4B and Table 2.3). Although analyses of the fungal communities in the eight soils showed that only three soils were undergoing directional changes (B, V an L soils), the overall result based on the simultaneous analysis of all soils yielded statistically significant regression lines (slope of 0.638, $P < 0.05$, Fig. 2.4C). None of the soil parameters measured had significant effects on the rates of change of bacterial and fungal communities. Significant and contrasting relationships were observed between the TLA slopes and the H′ values of archaeal ($r = +0.629$, $P < 0.05$) and bacterial ($r = -0.523$, $P < 0.05$) communities, but not of fungal communities.

Discussion

Temporal variation in the abundance of soil microbial communities

In our study, population sizes of archaea, bacteria and fungi, estimated using quantitative PCR, were found to be within the range observed in other soil systems (Ochsenreiter *et al.*, 2003; Bailey *et al.*, 2002). Quantitative PCR of soil DNA, as any PCR based approach, has its inherent limitations, given the known biases of soil DNA extraction, PCR and the core genes used as proxies for the three microbial communities. However, the method is highly reproducible and sensitive, enabling to quantify abundance changes across temporal and spatial scales. Moreover, in this study multiple runs were performed. In our calculations, we also took into account the efficiency and amount of extracted DNA from the soil samples. Therefore, we argue that our results are representative reflections of the fluctuations observed between different times, rather than pure noise.

A high abundance of crenarchaeota in soils has been previously observed (Kemnitz *et al.*, 2007; Ochsenreiter *et al.*, 2003), possibly indicating a crucial functional role for such organisms in agricultural soils. Furthermore, the bacterial abundance was often higher across soils than the fungal abundance (except at the end of 2009 and the end of 2011), supporting the finding that bacterial: fungal (B:F) ratios are quite high in agricultural or grassland soils as compared to forest soils, for instance (Bailey *et al.*, 2002; Bossuyt *et al.*, 2001; Högberg *et al.*, 2007; Treseder, 2004). These comparisons are very important in the context of whether soils are thought of a being fungal (more "natural") or bacterial (more highly cultivated) dominated. Indeed, such elevated B:F ratio's may also reflect anthropogenic disturbances due to agricultural practices.

The variations in microbial abundances could be explained by several parameters, depending on the target group. Soil pH and nitrate explained more than 75% of the variation in archaeal abundance. Previous studies have reported negative effects of pH on group 1.1c Crenarchaeota (Lethovirta *et al.*, 2009) in

acid forest soils, and negative relationships between nitrate and archaeal abundance (Bates *et al.*, 2011). The positive correlations between archaeal abundance and soil pH observed here suggest that our soils might be dominated by crenarchaeal species that are tuned to conditions of higher soil pH (7.0-7.5) (Bengston *et al.* 2012), which may be linked to the long agricultural history of the plots studied here.

Interestingly, the bacterial abundances didn't respond to soil pH or any other measured abiotic parameter, although several studies have reported pH as the main determinant of bacterial community composition (Fierer and Jackson, 2006; Lauber *et al.*, 2008). It has been shown that some specific bacterial taxa decrease or increase with changing pH, for instance members of the *Acidobacteria* and *Actinobacteria* (Lauber *et al.*, 2009). Although pH may have driven changes in the relative abundance of some bacterial classes, the abundance of total bacteria remained quite constant in the different pH ranges, indicating that the carrying capacity of the soil was not strongly affected by pH. Fungal abundance was also not affected by pH, but this was expected since the pH range in our soils was within the (wide) pH optimum for this group, often covering 5–9 pH units without significant inhibition of growth (Wheeler *et al.*, 1991; Nevarez *et al.*, 2009). We also observed that when conditions apparently favored increases in fungal abundance, archaeal abundance decreased, suggesting that fungi and archaea might compete for similar niches. Nonetheless, fungal abundance was positively affected by OM content, which is consistent with the saprophytic status of most fungi (de Boer *et al.*, 20065).

Temporal variation in α-diversity

None of the soil parameters measured in this study was able to explain the patterns of α-diversity observed for bacteria and fungi. It might be that the taxonomic scale was too broad and a deeper analysis would allow a better understanding of the observed patterns, as observed by Rasche *et al.* (Rasche *et al.*, 2011). With PCR-DGGE, only the most abundant taxa, comprising more than 0.1-1% of the community, can be detected. In other words, only the most abundant organisms are within PCR-DGGE roaming space. Because of these caveats, the parameters calculated from PCR-DGGE fingerprints and correlations based thereon should be interpreted as indications and not as absolute conclusions.

Archaeal α-diversity, on the other hand, was shown to respond to nitrate and OM levels. Nitrate had opposite effects on archaeal richness and diversity, depicting a community that responds to increasing nitrate with an increase of richness but a great decrease of evenness, most likely indicating the outgrowth of previously undetectable OTUs. Thus, in addition to the strong negative effect on archaeal abundance observed by qPCR, nitrate availability seems to be a cru-

cial factor determining archaeal community structure. The positive correlation between archaeal diversity and soil OM content indicate that the OM provides a substantial fraction of carbon to the local archaeal communities. Recently, genomic analyses of *Crenarchaeum symbiosum* and *Nitrosopumilus maritimus* suggested that these organisms are capable of mixotrophy (Hallam *et al.*, 2006; Walker *et al.*, 2010), and that group 1.1c Crenarchaeota are able to grown on methanol and methane (Bomberg and Tomonen, 2007). This indicates that archaea might not be solely sustained by ammonia oxidation (Ouverney and Fuhrman , 2000).

Variation in β-diversity over time (species turnover)

To assess how dynamic each soil microbial group was over time, we calculated the temporal β-diversity (average Bray Curtis dissimilarity) for each soil over time and for each microbial group. We observed a higher temporal β-diversity for archaea than for bacteria and fungi across all soils. This indicates that the archaeal communities are much more dynamic than the bacterial or fungal ones along a time gradient. These differences are probably due to the differential physiologies and sensitivities to environmental insults of these microorganisms. It has been shown that changes in temperature and moisture (Rasche *et al.*, 2011; Tourna *et al.*, 2008), and resource availability due to seasonal variation (Rasche *et al.*, 2011) can affect soil archaeal as well as bacterial communities. Moreover, a clear pattern was observed for bacterial β-diversity in the metacommunity of 17 rock pools, with higher variations during summer and lower during autumn (Langenheder *et al.*, 2012). The temporal variations of archaeal and bacterial communities were also higher in the clayey soils than in sandy ones, suggesting that the latter harbors more dynamic communities.

One main finding of this study is that, although the β-diversity patterns of the three microbial domains investigated are related with the same set of abiotic factors, the total percentage of variation able to explain those patterns was much higher for archaeal (45.0%) than for bacterial (6.6%) or fungal (6.9%) communities. This suggests that the archaeal communities might be much more sensitive to environmental changes than the bacterial or fungal ones. Based on these results, we hypothesize that the archaeal communities of agricultural soils with a long history of N-fertilization are more sensitive to disturbances than the corresponding bacterial or fungal communities.

Quantification of β-diversity

To be able to quantify community dynamics, allowing comparisons and providing a general overview of long-term trends in the complex soil system, we used the slopes obtained from TLA. TLA has been intensively used to identify directional changes and to quantify temporal dynamics of macroorganisms (Thibault *et al.*, 2004; Baez *et al.*, 2006; Collins and Smith, 2006; Feeley *et al.*, 2011;

Flohre *et al.*, 2011), but very few studies have focused on microorganisms. Although the TLA slopes for archaea and bacteria were small, they were significantly different from neutral. Clearly, even small changes can be part of a long-term trend. On the contrary, changes in the fungal communities were non-significant, suggesting stochastic species dynamics.

Changes in environmental variables within soil sites determine how time affects turnover (β-diversity), as different microbial interactions are favored if prevailing conditions change (Chesson and Huntly, 1997). Only archaeal communities responded to changes in environmental parameters, being strongly correlated with nitrogen availability and with the degree of temporal variation quantified by TLA. This might suggest that at some level, strongly deterministic processes are acting on the archaeal, but not on the bacterial and fungal communities in these soils. Another explanation is that archaea are much more limited in their ecoversatility, whereas bacteria and fungi are highly functionally redundant. The observed relation between bacterial richness and TLA slopes, e.g. high turnover at low richness, was also noticed in a study of the distribution of British birds (Lennon *et al.*, 2001). The authors discuss that low species richness areas tend to have relatively more random mixtures of species than high species richness areas. The relation observed between archaeal turnover and species richness suggests a less random distribution of species, caused mainly by nitrate contents.

In this study we demonstrate that changes in the community composition of bacteria and fungi could be linked to both environmental and biotic factors (e.g. species-species interactions), as their (α-) diversity co-varied significantly with their β-diversity over the time period of study. Conversely, for archaea no significant correlation was observed between α- and β-diversity, and the community shifts were mainly driven by the surrounding environment, mostly by the effects of soil pH and nitrate concentrations. This might indicate that changes in archaeal community are mostly driven by environmental factors, as previously observed by Zinger *et al.* (Zinger *et al.*, 2011) in a study on the patterns of archaeal, bacterial and fungal communities in an alpine landscape. Furthermore, we propose that different environmental and biological mechanisms act on each microbial niche. A more comprehensive understanding of the rules governing these important soil microorganisms will require additional field work as well as microcosm experiments to identify the key environmental and biotic factors driving the assemblage of these communities.

Ethic statement

No specific permits were required for the described field studies. The locations are not protected. The field studies did not involve endangered or protected species.

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Table S1. Soil chemical parameters measured in this study.

	Apr 09	Jun 09	Sep 09	Nov 09	Apr 10	Jun 10	Oct 10	Feb 11	Apr 11	Jun 11	Sep 11
pH											
B		4.37	4.26	4.42	4.23	4.59	4.38	4.39	4.23	4.41	4.38
V	4.43	4.59	4.82	5.13	4.33	4.50	4.82	5.10	4.33	4.42	4.82
D	5.04	5.26	5.05	5.49	5.03	5.06	4.70	5.46	5.03	5.05	4.70
W		4.57	4.63	5.03	4.37	4.70	4.73	5.00	4.37	4.54	4.73
K		7.45	7.56	7.53	7.41	7.41	7.43	7.50	7.41	7.41	7.43
S	5.73	5.76	5.68	5.49	5.14	5.40	5.38	5.46	5.14	5.27	5.38
G		7.45	7.54	7.64	7.17	7.05	7.38	7.61	7.17	7.11	7.38
L	7.45	7.38	7.53	7.71	7.21	7.38	7.36	7.68	7.21	7.30	7.36
Mean	5.66	5.85	5.88	6.06	5.61	5.76	5.77	6.03	5.61	5.69	5.77
CV (%)	19.95	21.97	22.77	20.76	23.47	21.00	22.16	20.87	23.47	22.19	22.16
$N\text{-}NO_3^-$ (mg/kg)											
B		165.11	19.02	48.80	24.45	103.64	13.37	13.41	23.42	109.45	1.34
V	86.60	119.45	99.29	34.50	79.57	99.16	45.75	6.65	85.91	113.23	4.60
D	67.17	69.80	59.86	103.00	67.91	68.53	45.16	3.63	30.77	76.15	1.33
W		44.18	24.40	0.40	39.51	21.73	6.52	10.84	69.39	13.55	2.41
K		22.89	10.23	7.50	43.58	14.82	15.42	2.50	143.18	11.73	5.28
S	126.50	62.43	19.74	94.50	103.38	56.51	28.09	13.67	140.67	75.83	0.76
G		15.72	20.29	14.60	59.03	12.17	18.62	2.24	71.59	16.41	1.87
L	22.15	9.12	10.12	49.10	33.59	4.30	20.30	3.35	135.00	4.29	2.04
Mean	75.61	63.59	32.87	44.05	56.38	47.61	24.15	7.04	87.49	52.58	2.45
CV (%)	49.67	80.11	88.47	81.33	43.78	78.56	56.15	65.12	51.26	81.91	61.92
$N\text{-}NH_4^+$ (mg/kg)											
B		12.09	2.66	16.80	9.17	12.83	4.52	20.81	40.68	11.49	18.02
V	17.51	20.15	2.34	8.37	22.34	24.90	21.14	21.14	23.62	9.33	8.35
D	12.71	11.32	1.26	22.37	21.93	15.79	14.60	14.60	8.71	11.80	27.06
W		11.05	2.86	6.60	10.56	10.55	8.08	8.08	3.91	6.28	8.54
K		9.71	1.03	3.50	8.79	6.73	7.65	21.05	4.10	5.29	4.20
S	14.88	11.03	2.73	25.20	19.89	10.90	5.34	9.04	6.07	2.32	29.76
G		8.86	1.16	3.60	20.29	21.61	4.26	7.82	2.57	3.58	35.55
L	8.20	9.36	1.37	29.50	14.99	10.12	5.30	6.20	6.99	3.13	4.45
Mean	13.33	11.69	1.93	14.49	16.00	14.18	8.86	13.59	12.08	6.65	16.99
CV (%)	25.61	28.68	38.51	66.65	34.12	41.01	63.23	45.42	103.27	53.05	68.29
OM (%)											
B		3.64	3.67	4.15	4.95	3.47	3.61	4.95	3.47	3.61	3.67
V	12.97	14.19	20.01	24.70	15.26	15.68	19.86	15.26	15.68	19.86	20.01
D	2.85	3.44	2.97	3.70	2.61	3.25	2.63	2.61	3.25	2.63	2.97
W		3.34	3.74	6.90	4.61	2.58	3.63	4.61	2.58	3.63	3.74
K		2.72	2.64	3.34	6.58	3.31	2.69	6.58	3.31	2.69	2.64
S	5.99	5.60	4.00	6.37	6.52	5.01	4.43	6.52	5.01	4.43	4.00
G		4.87	5.41	5.60	6.37	4.34	5.47	6.37	4.34	5.47	5.41
L	2.76	2.63	2.84	4.38	2.77	2.51	3.15	2.77	2.51	3.15	2.84
Mean	6.14	5.05	5.66	7.39	6.21	5.02	5.68	6.21	5.02	5.68	5.66
CV (%)	67.58	70.90	96.92	89.95	60.04	81.79	95.52	60.04	81.79	95.52	96.92

Table S2. PCR mixtures for real time quantification of Archaeal 16S rDNA, Bacterial 16S rDNA and Fungal ITS region.

Real time PCR primers (5′- 3′)	PCR mixtures	Thermal conditions
Archaeal 16S		
771F (ACGGTGAGGGATGAAAGCT) (Ochsenreiter *et al.*, 2003) 957R (CGGCGTTGACTCCAATTG) (Ochsenreiter *et al.*, 2003)	12.5µl Power Sybr Green PCR Master mix, 0.5ul BSA (20mg/ml), 0.8µM each primer and 2ul DNA template	95°C 10 min, 1 cycle 95°C for 30 s, 54°C for 30 s, 72°C for 30 s, 39 cycles
Bacterial 16S		
16SFP (GGTAGTCYAYGCMSTAAACG) (Bach *et al.*, 2002) 16SRP (GACARCCATGCASCACCTG) (Bach *et al.*, 2002)	12.5µl Power Sybr Green PCR Master mix, 0.5ul BSA (20mg/ml), 0.8µM each primer and 2ul DNA template	95°C 10 min, 1 cycle 95°C for 27s, 62°C for 1 min, 72°C for 30s, 39 cycles
Fungi		
5,8S (CGCTGCGTTCTTCATCG) (Vigalys *et al.*, 1990) ITS1f (TCCGTAGGTGAACCTGCGG) (Gardes and Bruns, 1993)	12.5µl Power Sybr Green PCR Master mix, 0.5ul BSA (20mg/ml), 0.8µM each primer and 2ul DNA template	95°C 10 min, 1 cycle 95°C for 1 min, 53°C for 30s, 72°C for 1 min, 40 cycles

Table S3. PCR mixtures for DGGE analysis of Archaeal 16S rDNA, Bacterial 16S rDNA and Fungal ITS region

PCR-DGGE primers (5′- 3′)	PCR mixtures	Thermal conditions
***Archaeal 16S* rDNA**		
A2F - TTCCGGTTGATCCYGCCGGA (DeLong, 1992) U1406F - ACGGGCGGTGTGTRC (Koga *et al*., 1993)	0.2mM dNTPs, 1x buffer (Roche), 0.25µl BS, 0.5µl DMSO, 0.5µM each primer, 0.2U Taq polymerase (Roche)	95ºC 5 min 94ºC 1 min, 57.5ºC 30 s, 72ºC 4 min, 35 cycles final ext. of 72ºC 7 min
ARC344 (*ACGGGGCGCAGCAG GCGCGA) (Bano *et al*., 2004) 517r (ATTACCGCGGCTGCTGG) (Bano *et al*., 2004)	0.2mM dNTPs, 1x buffer (Roche), 0.4µM each primer, 0.5U Taq polymerase (Roche)	94ºC 5min 94ºC 45s, 65ºC-62ºC 45s, 7 cycles, 72ºC 30s 94ºC 45s, 62ºC-55ºC 45s, 6 cycles, 72ºC 30s 94ºC 45s, 55ºC 45s, 30 cycles, 72ºC 30s 72ºC 10 min
Bacterial 16S		
F968 (*AACGCGAAGAACCTTAC) (Gomes *et al*., 2001) R1401.1b (CGGTGTGTACAAGAC CCGGGAACG) (Brons and van Elsas, 2008)	0.2mM dNTPs, 3.75mM $MgCl_2$, 1x buffer (Bioline), 1% formamide, 0.2µM each primer, 2.5U Taq polymerase (Bioline)	95ºC 5 min 60ºC 1′(- 1º /cycle, until 55ºC); 72ºC 2 min 10 cycles 94ºC 1 min, 55ºC 1 min, 72ºC 2 min, 20 cycles 72ºC 10 min
ITS region		
EF4 (GGAAGGGRTGTATTTATTAG) (Smit *et al*., 1999) ITS4 (TCCTCCGCTTATTGATATGC) (White *et al*., 1990)	0.2 dNTPs, 2.0 mM $MgCl_2$, 1x buffer (Bioline), 0.025µl T4 gene protein, 0.4µM each primer, 2.5U Taq polymerase (Bioline)	94ºC 5min 94ºC 30s, 55ºC 30s, 72ºC 1 min 30s, 34 cycles 72ºC 5 min
ITS1f (*CTTGGTCATTTAGAGGA AGTA) (Gardes and Bruns, 1993) ITS-2 (GCTGCGTTCTTCATCGAT GC) (White *et al*., 1990)	0.25mM dNTPs, 2.0mM $MgCl_2$, 1x buffer (Bioline), 0.4µM each primer, 2.5U Taq polymerase (Bioline)	94º C 5min 94º C 30s, 55º C 30s, 72ºC 30s, 34 cycles 72º C 5 min

* Means that a GC-clamp is present (Muyzer *et al*., 2001)

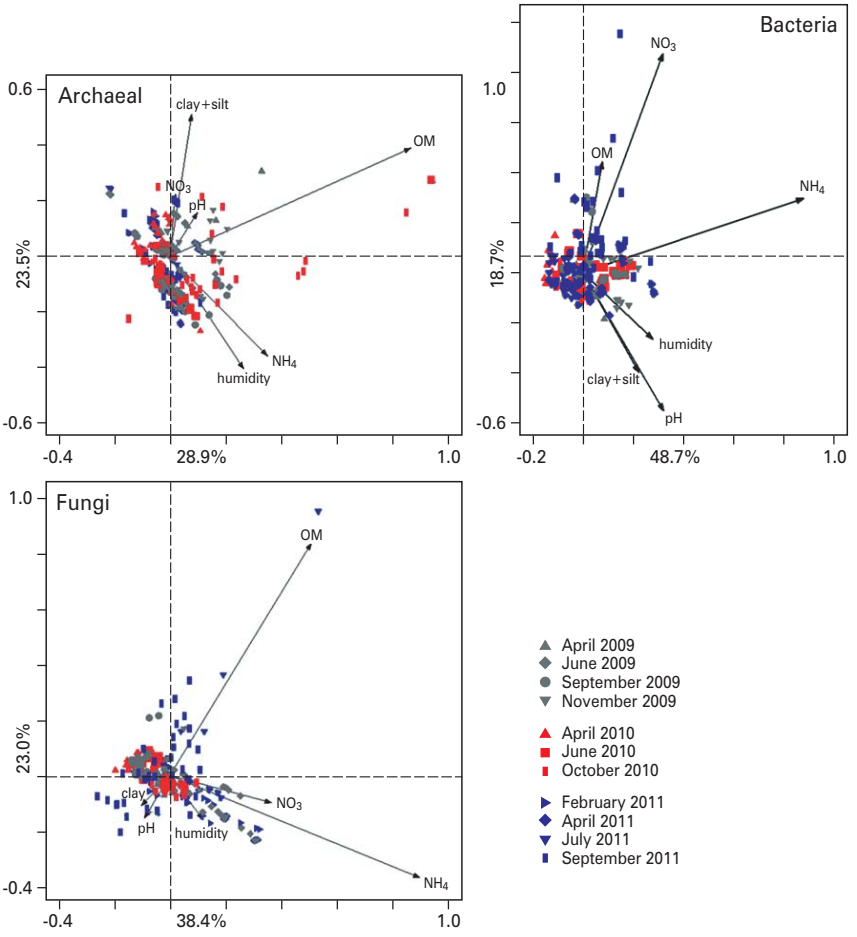

Figure S1. Canonical Correspondence Analysis (CCA) of archaeal, bacterial and fungal similarity matrices and vector fitting of the environmental variables.

Chapter 3

Seasonal variations in diversity and abundance of diazotrophic communities across soils

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Abstract

The N-fixing community is a key functional community in soil, as it replenishes the pool of biologically available nitrogen that is lost to the atmosphere via denitrification. We characterized the structure and dynamic changes in diazotrophic communities, based on the nifH gene, across eight different representative Dutch soils during one complete growing season, to evaluate the amplitude of the natural variation in abundance and diversity, and identify possible relationships with abiotic factors. Overall, our results indicate that soil type is the main factor influencing the N-fixing communities, with clay soils being more abundant and diverse than sandy soils. On average, the amplitude of variation in community size as well as the range-weighted richness was also found to be higher in the clay soils. These results indicate that nitrogen fixing communities associated with sandy and clay soil have distinct normal operating ranges (amplitude of variation) and suggest that the diazotrophic communities associated with clay soil might be more sensitive to fluctuations associated with the season and agricultural practices. Moreover, soil characteristics such as ammonium content, pH and texture most strongly correlated with the variations observed in the diversity, size and structure of nitrogen fixing communities, whose relative importance was determined across a temporal and spatial scale.

Introduction

Biological nitrogen fixation (BNF) is the reduction of atmospheric N_2 gas to biologically available ammonium, catalyzed by the enzyme nitrogenase (Postgate, 1998). It is performed by phylogenetically diverse groups of prokaryotic microorganisms which harbor the *nif*H gene, one of the genes coding for the structural part of nitrogenase. BNF is important in terrestrial ecosystems as it replenishes the pool of biologically available nitrogen that is lost to the atmosphere via anaerobic ammonium oxidation and denitrification (Capone and Knapp, 2007). Nitrogen-fixing bacteria are important regulators of plant productivity because plants cannot fix atmospheric N and because N is, together with phosphorus and potassium, the main element that limits plant productivity (Chapin, 1980). Although the majority of N fixation is carried out by bacteria that live in association with plants, free-living diazotrophs in soils have been shown to be important contributors to the N budgets in several ecosystems, reaching up to 60 kg ha^{-1} $year^{-1}$ (Cleveland *et al.*, 1999). Duc *et al.* (2009) studied the diversity of free-living diazotrophs in the soils of the Damma glacier (Swiss Central Alps), and also measured the potential asymbiotic activity using the acetylene reduction assay, revealing a high diversity of *nif*H gene sequences and illustrating the importance of free-living diazotrophs and their potential contribution to the nitrogen input in this environment.

Symbiotic nitrogen-fixing bacteria are known to be highly sensitive to perturbation (Doran and Safley, 1997). Indeed, several environmental factors have been suggested to influence N fixation in soils, including soil moisture, oxygen, pH, carbon quantity and quality, nitrogen availability (Hsu and Buckley, 2009), soil texture and aggregate size (Poly *et al.*, 2001b), and clay content (Roper and Smith, 1991). For instance, Wakelin *et al.* (2007) showed that retention of stubble in soil approximately doubled the abundance of *nif*H genes. This was not affected by the application of an N source such as urea. In another experiment, the addition of nitrogen fertilizers led to rapid changes (within 15 days) in the community structure of *nif*H harboring bacteria in association with rice roots (Tan *et al.*, 2003). Plants are known to affect free-living N-fixing activities in soil, which was correlated with the grass (Patra *et al.*, 2006), as well as the diazotrophic community structure, as it was shown that different cultivars of sorghum (*Sorghum bicolor*) select for different *nif*H communities (Coelho *et al.*, 2008). The community structure of diazotrophic bacteria is also likely to be affected by soil type, as different soil fractions, such as sand-sized and silt-sized ones, harbored different N fixing populations (Gros *et al.*, 2006). Moreover, this was also true among microenvironments (Poly *et al.*, 2001b), defined on the basis of the size of constituent particles, organic carbon and clay content. Temporal variations were shown to affect the level of cultivable diazotrophic population in soils, with highest numbers in autumn/winter/early spring and with low counts in

summer (Mergel *et al.*, 2001). However, the low number of cultured bacteria found does not allow further generalization of these data

In general, soil microbial communities are affected by a magnitude of natural fluctuations (such as temperature, plant growth or rainfall), and also agricultural practices, such as plowing (Buckley *et al.*, 2001; Clegg *et al.*, 2003). The most important factors that determine the diversity of microorganisms in soil are soil type and soil edaphic factors, plant type and soil management practices (van Overbeek and van Elsas, 2008). Nevertheless, the extent to which these factors affect the amplitude of variation of microbial communities remains unclear. In order to understand the functioning of microbial communities and their resilience to external changes, a key issue is to assess the community composition, quantify individual microbial population sizes, and study fluctuations thereof (van Elsas *et al.*, 2000; Hartmann and Widmer, 2006), as these fluctuations will form the reference against which external disturbances can be reflected upon. However, a thorough description of the natural variation of the nitrogen fixing community in a wide range of soils is still missing.

In the present study, we characterized the dynamics and variation of the diazotrophic community structure using denaturing gradient gel electrophoresis (DGGE) and clone libraries. Furthermore, we used real-time PCR to assess community size. Eight different representative soils (both in terms of soil properties and biological factors) were used. Our main hypotheses were: (1) diazotrophic assemblages are responsive to biotic and abiotic parameters, ultimately affecting functional aspects; (2) soil type is the major factor influencing the structure and abundance of the N-fixing community. This study will provide insights into basic parameters that influence the N-fixing community in our soils, which will allow us to set the baseline of this important functional group, across soil types, land use types and seasonal changes.

Material and Methods

Experimental sites and soil sampling

Eight fields across the Netherlands were selected based on their soil properties and cropping systems, four sandy (B, V, D, W) and four clay soils (S, K, G, L) (Table 3.1). These are all potato fields, subjected to crop rotation with non-leguminous plants, except for W, which is a permanent grassland soil. Bulk soil samples were collected four times over a growth season plus fall: April, June, September and November 2009. April and June are associated with the growing season, September represents the end of the growing season and November is associated with the raining season and no plants were present in the field. All eight soils were sampled on the same day at each sampling time. For each soil, four replicates were taken. Each replicate consisted of 10 sub-samples (15–20 cm

deep) collected between plots, away from the roots, with a spade. A total of two kilograms of each soil (0.5 kg per replicate) were thus collected in plastic bags and thoroughly homogenized before further processing in the lab.

Soil chemical analysis

Dried (24 h at 40oC) soil samples were ground, sieved through a 2 mm mesh and analyzed. The pH was measured in water suspension 1: 4.5 (g/v) with an Inolab Level 1 pH-meter (WTW GmbH, Weilheim, Germany). Nitrate (NO_3^-) and ammonium (NH_4^+) were determined calorimetrically in a solution of 0.01 M $CaCl_2$ with an Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, New York). Dissolved organic carbon (DOC) was measured by a carbon analyzer in a soil extract of 0.01 M $CaCl_2$. Water content was measured by comparison of fresh and dried (48 h at 65°C) weight of samples. Organic matter (OM) content is calculated as the difference between the initial and final sample weights measured after 2 hours at 550°C, divided by the initial sample weight times 100%.

Nucleic acid extraction

For extraction of soil DNA, the PowerSoil DNA extraction kit (Mo Bio Laboratories Inc., NY) was used with 0.5 g of soil, according to the manufacturer's instructions, with the exception that glass beads (diameter 0.1 mm; 0.25 g) were added to the soil slurries. The cells were disrupted by bead beating (mini-bead beater; BioSpec Products, United States) three times for 60 s. To assess the quantity and purity, the crude DNA extracts were run on 1.5% agarose gels at 90 V for 1 h in 0.5X Tris-acetate-EDTA (TAE) buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA; pH 8.0) using a fixed amount (5 µl) of a 1-kb DNA ladder (Promega, Leiden, Netherlands) as the molecular size and quantity marker. After staining with ethidium bromide, DNA quality was determined based on the degree of DNA shearing (average molecular size) as well as the amounts of coextracted compounds. Good quality DNA was obtained from all soils and yields were between 0.5 µg and 3.2 µg DNA per g of soil.

PCR amplification of *nif*H gene for DGGE analysis

PCR of *nif*H genes was conducted using a nested PCR according to Diallo *et al.* (2004), where a detailed protocol is described. The primers used in the first PCR reaction were FPGH19 (5'-TACGGCAARGGTGGNATHG-3'; Simonet *et al.*, 1991) and PolR (5'- ATSGCCATCATYTCRCCGGA-3'; Poly *et al.*, 2001a), and 2 µl of the first PCR product was used as the template in the second reaction with primers PolF containing a GC clamp (5'-TGCGAYCCSAARGCBGACTC-3') and AQER (5'-GACGATGTAGATYTCCTG-3') (both Poly et al, 2001a). The concentration of the PCR products was determined by 1.5% (w/v) agarose TAE gel, staining with ethidium bromide to confirm product integrity and size by

comparing with a molecular weight marker (Smart ladder; Eurogentec). All soils were PCR-amplifiable, resulting in one single band of the expected size (360bp).

DGGE systems for detection of *nif*H

DGGE profiles were generated with the Ingeny Phor-U system (Ingeny International, Goes, The Netherlands). PCR products (250-300 ng/ lane) were loaded onto 6% (w/v) polyacrylamide gels, 1 mm thick, in 0.5X TAE buffer with a 40-65% denaturant gradient (100% denaturant corresponded to 7 M urea and 40% (v/v) deionized formamide) to separate the generated amplicons. Electrophoresis was performed at a constant voltage of 100 V for 16 h at 60ºC. The gels were stained for 60 min in 0.5X TAE buffer with SYBR Gold (final concentration 0.5 µg/liter; Invitrogen, Breda, The Netherlands). Images of the gels were obtained with Imagemaster VDS (Amersham Biosciences, Buckinghamshire, United Kingdom) and stored as TIFF files.

Computer-assisted analysis of DGGE fingerprinting

DGGE patterns were compared by clustering the different lanes by Pearson's correlation coefficient implemented in the GelCompar II software (Applied Maths, Sint-Martens Latem, Belgium), using the unweighted-pair group method with arithmetic mean, rolling-disk background subtraction, and no optimization (Kropf *et al.*, 2004; Rademaker, *et al.*, 1999). Range-weighted richness (Rr) values (Marzoratti *et al.*, 2008) were calculated based on the total number of bands (N), and the denaturing gradient comprised between the first and the last band of the pattern (Dg), according to the following equation: $Rr = N^2 \times Dg$. To assess the interspecies abundance ratios, Pareto-Lorenz curve distribution patterns of the *nif*H DGGE profiles were plotted based on the numbers of bands and their intensities to visualize the functional organization (Fo) of the diazotrophic community over time (Mertens *et al.*, 2005, Marzoratti *et al.*, 2008). Data derived on the basis of Jaccard correlation (a band-based analysis) were used for principal component analysis (PCA) using CANOCO (version 4.52, Wageningen, The Netherlands). The matrix of similarities was also used to perform moving window analysis (Wittebolle *et al.*, 2005) and to calculate the percentage of change, as *change% = 100 – similarity%*.

Quantification of the N-fixing community

To quantify the number of copies of the *nif*H gene, the primers FPGH19 (Simonet *et al.*, 1991) and PolR (Poly *et al.*, 2001a) were used. The PCR mixture and thermal cycling conditions are described in Taketani *et al.* (2009). Absolute quantification of the *nif*H gene was carried out in four replicates on the ABI Prism 7300 Cycler (Applied Biosystems, Germany). The specificity of the amplification products was confirmed by melting curve analysis, and the expected

sizes (450bp) of the amplified fragments were checked in a 1.5% agarose gel stained with ethidium bromide. Standard curves were obtained using serial dilutions of the *Escherichia coli* derived vector plasmid JM 109 (Promega, Madison, WI, EUA) containing a cloned *nif*H gene from *Bradyrhizobium liaoningense*, using 10^7 to 10^2 gene copy numbers/µl. The efficiency was calculated by using the formula Eff = [10(-1/slope) -1]. Two independent qPCRs were performed for all samples and the results were similar. To test for inhibition in the PCR reactions, DNA extracted from soil was diluted and mixed with known amount of standard DNA before qPCR. The Ct values obtained for the standard DNA did not change in the presence of diluted soil DNA, indicating the absence of severe inhibition.

Construction of *nif*H clone libraries

DNA extracted from soil collected in June was used to construct *nif*H-based clone libraries for each of the eight soils. The four replicates of each soil were used in PCR amplifications with primers PolF and AQER (both Poly et al, 2001a). A detailed protocol is described in Diallo *et al.* (2004). The products (size: 320 bp) were pooled per soil and gel-purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, EUA). The purified PCR products were then inserted into the vector pGEM-T-Easy (Promega, Madison, WI, EUA) and introduced into competent *Escherichia coli* JM 109 cells in accordance with the manufacturer's instructions (Promega, Madison, WI, EUA). Clones containing the insert were sequenced using an Applied Biosystems 3730 XL DNA Analyzer at LGC Genomics GmbH (Berlin, Germany).

Phylogenetic analysis

The *nif*H gene sequences were compared with sequences in GenBank using nucleotide-nucleotide BLAST (BLAST-N) to obtain the nearest phylogenetic neighbors (www.ncbi.nlm.nih.gov/BLAST). Sequences were checked for chimera using Bellerophon v.3 (http://greengenes.lbl.gov) (Huber *et al.*, 2004). Sequences were then processed in Mega 4 (Tamura *et al.*, 2007), translated, and the deduced amino acid sequences were aligned using Clustal W (Jeanmougin, 1998). Phylogenetic trees were constructed using the neighbor-joining method with the Jones-Taylor-Thornton model for amino acid substitution. In addition, we performed maximum-likelihood analyses (Olsen *et al.*, 1994). Both trees showed similar topologies. Bootstrapping (500 replicates) was used to estimate the reliability of the phylogenetic reconstructions. The program DOTUR (Distance-based OTU and richness) (Schloss and Handelsman, 2008); http://www.plantpath.wisc.edu/fac/joh/DOTUR.html) was used to create rarefaction curves where a conservative OTU cutoff of 97% similarity was used to determine the Shannon diversity index, as well as the bias-corrected Chao1 estimator of richness. Differences in the community structures of *nif*H clone

Table 3.1. Soil characteristics measured in this study.

Soil location	pH				OM (%)				DOC (mg/kg)				$N\text{-}NO_3^-$ (mg/Kg)				$N\text{-}NH_4^+$ (mg/Kg)			
	A	J	S	N	A	J	S	N	A	J	S	N	A	J	S	N	A	J	S	N
Sandy																				
Buinen (B)		4.4	4.3	4.4		3.6	3.6	4.1		52.3	47.1	75.9		165.1	19.0	48.7		12.1	2.6	16.7
Valthermond (V)	4.4	4.6	4.8	5.1	12.97	14.2	20.0	24.6	186.9	193.7	277.8	225	86.6	119.5	99.3	34.4	17.5	20.2	2.3	8.9
Droevendaal (D)	5.0	5.3	5.1	5.5	2.85	3.4	2.9	3.6	65.6	70.9	68.2	94.3	67.2	69.8	59.8	102.0	12.7	11.3	1.3	22.9
Wildekamp (W)		4.6	4.6	5.0		3.3	3.7	6.8		80.6	91.8	278.0		44.2	24.4	3.0		11.0	2.8	6.8
Clay																				
Kollumerward (K)		7.5	7.6	7.5		2.7	2.6	3.3		60.5	52.8	81.9		22.8	10.2	7.4		9.7	1.0	3.4
Steenharst (S)	5.7	5.7	5.7	5.5	5.99	5.6	4.0	6.4	171.1	158.7	161.0	204.0	126.5	62.4	19.7	94.4	14.8	11.0	2.7	25.1
Grebbedijk (G)		7.5	7.5	7.6		4.8	5.4	5.6		108.5	90.4	101.0		15.7	20.3	14.8		8.8	1.2	3.5
Lelystad (L)	7.5	7.4	7.5	7.7	2.76	2.6	2.8	4.3	60.7	57.0	57.9	121.0	22.15	9.1	10.1	49.0	8.2	9.4	1.4	29.4

A = April; J = June; S = September and N = November. In April only four soils were analyzed. OM = organic matter; DOC = dissolved organic carbon; $N\text{-}NO_3^-$ = nitrate and $N\text{-}NH_4^+$ = ammonium.

Numbers are average of three replicates.

libraries were analyzed with UniFrac (Lozupone *et al.*, 2006), using maximum-likelihood-based trees.

Statistical analysis

Physico-chemical and biological variables were checked for normality and transformed when necessary. Differences in these variables between sandy and clay soils, among all eight soils, and over time were assessed with independent sample t-tests. Bonferroni, Hochberg and False Discovery Rate correction methods were implemented for multiple t-tests to avoid the change of type I error. Multiple regression analyses were conducted on normalized data to avoid possible nonlinear relations (SAS® system for Windows version 8.02, SAS Institute Inc, Cary, NC, USA, 2001). The following parameters were included in the analysis: diversity (Shannon diversity index *H'* of clone libraries) and abundance (A) of N-fixing community; nitrate in mg kg^{-1} dw^{-1} (NO_3); ammonium in mg kg^{-1} (NH_4); pH; dissolved organic carbon in mg mg^{-1} (DOC) and organic matter in % (OM). Variables in the regression models were significant at the 0.1 level. Models were restricted to a maximum of two parameters. Multiple regressions were conducted for each soil separately.

Nucleotide sequence accession numbers

The sequences generated in this study have been deposited in the GenBank database under accession numbers HQ335394 - HQ336041.

Results

Physico-chemical characteristics of soils

Soil chemical analyses were performed across a season and all soils revealed significant differences between various parameters (Table 3.1). Regarding seasonal variations, there were significant ($P < 0.05$) differences in levels of NO_3^-, from June to September, and in levels of NH_4^+, from June to September and September to November. Small, but non significant, variations of pH, OM and DOC were found over time (Table 3.1). Considering soil type, pH was significantly higher ($P < 0.05$) in the clay (6.94 ± 0.11, $n = 12$) than in the sandy soils (4.78 ± 0.08, $n = 12$) during the whole period. The NO_3^- and NH_4^+ concentrations (mg/kg) were significantly higher in the sandy soils (NO_3^-: 75.14 ± 11.86; NH_4^+: 7.96 ± 0.66, $n = 12$) than the clay soils (NO_3^-: 21.31 ± 3.87; NH_4^+: 5.66 ± 0.22, $n = 12$), in the June and September only. In April and November, the differences were not significant. Although bulk soil was always analyzed, the crops present in the field over the season were barley (soils B and V), triticale (cross between wheat and rye; soil D), potato (soils K, G and L), and grass (soils W and S).

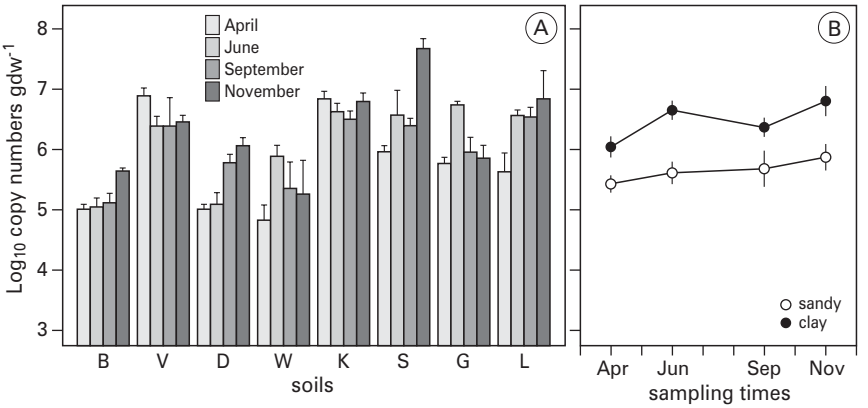


Figure 3.1. Real time quantification of *nif*H gene across eight soils. Absolute numbers for each sampling time (A) and average abundance values between sandy and clay soils (B). Soils used in this work: V, Valthermond; B, Buinen; W, Wildekamp; D, Droevendaal; K, Kollummerwaard; S, Steenharst; G, Grebbedijk; L, Lelystad. Bars are standard errors (A, n = 4; B, n = 16).

Abundance of *nif*H-harboring bacteria

Overall, the population sizes of nitrogen fixing bacteria, quantified by real-time PCR targeting the *nif*H gene, varied from 10^5 gene copies gdw^{-1} in April to 10^7 gene copies gdw^{-1} in November (Fig. 3.1A). Grouping of the soils according to their texture revealed that, on average, clay soils had higher *nif*H abundances (log 6.4 gene copies gdw^{-1}) than sandy ones (log 5.6 gene copies gdw^{-1}) ($P < 0.05$, n = 16) (Fig. 3.1B). Analyses across the season revealed that the *nif*H gene abundance in the sandy soils was significantly lower in April ($P < 0.05$) than in November, but no significant changes were found from June to September. The *nif*H gene abundance in the clay soils showed a higher variation, going significantly up from April to June, and significantly down from June to September (Fig. 3.1B).

Community structure analysis based on the *nif*H gene

In order to characterize the structure and dynamics of the N-fixing communities through time across different soils, we performed DGGE analyses based on the *nif*H gene. Analysis of the DGGE gels revealed that the numbers of bands per sample varied between 35 ± 1 and 14 ± 1, and decreased from April to November for both sandy and clay soils. The range-weighted richness decrease significantly from April (122 ± 10.4, n = 12) to November (26.5 ± 8.2, n = 12) (Fig. S1) being significantly higher in the sandy soil in June (Fig. 3.2). It is worthwhile mentioning that W soil (grassland) had, on average, the lowest Rr value. Multivariate analyses (PCA) of the *nif*H DGGE profiles showed that the profiles

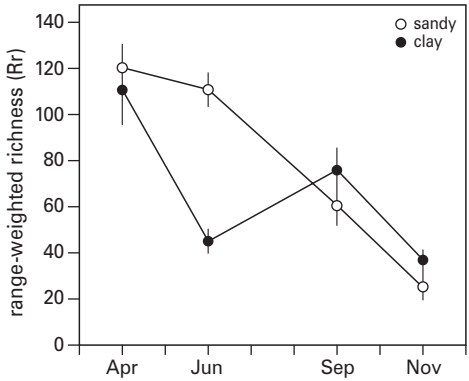


Figure 3.2. Average range-weighted richness (Rr) values from the sandy and clay soils and the fluctuations over time. Bars are standard errors (n = 12).

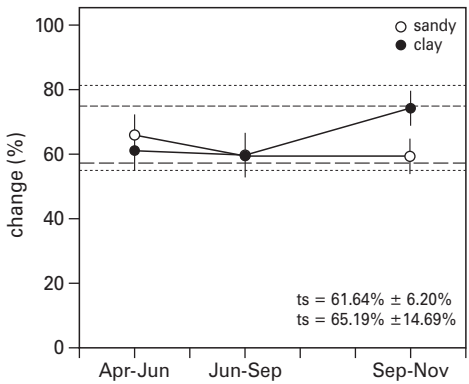


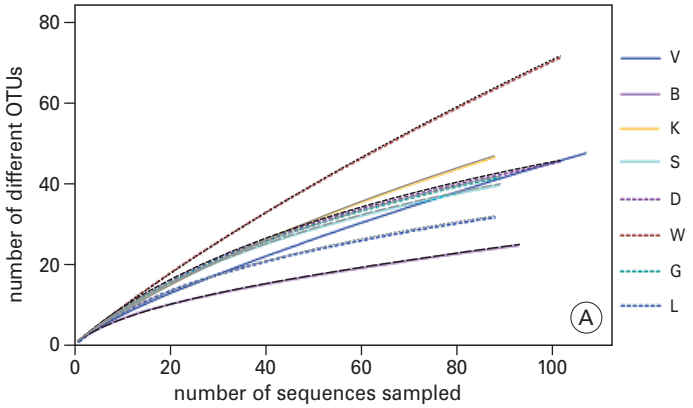
Figure 3.3. Moving window analysis based on DGGE profile of N-fixing communities. Each data point in the graph is in itself a comparison between two consecutive sampling times. Δt values (Wittebolle et al., 2005) were calculated as the averages and standard deviations for the respective change% values. Bars are standard errors (n = 12).

tend to cluster by soil type in April and June, along the first and second axes respectively (Fig. S2A and S2B). In September and November, the profiles formed a diffuse cluster, and no clear trend was observed (Fig. S2C and S2D).

The dynamics of N-fixing community were established with the moving window analysis and showed that all eight soils had pronounced changes through time, with amplitude of variations being higher in the clay soils (Δt_s = 65.19 ± 14.69%) coming to significance in November, compared to the sandy ones (Δt_s = 61.64 ± 6.20%) (Fig. 3.3). The functional organization was comparable, as 20% of the bands (number based) corresponded to, on average, 45% of the cumulative band intensities (Fig. S3).

Diversity of *nif*H-harbouring bacteria

In order to identify and characterize the dominant types of nitrogen-fixing bacteria across different soils, we constructed one clone library per soil, based on the *nif*H gene, using the samples collected in June 2009, by pooling the *nif*H products obtained from 4 replicates prior to cloning. The results first showed that the similarity of the deduced sequences to known *nif*H sequences (database) varied from 81 to 100%. Around 11% of the sequences remained unclassified (< 89% similarity), and these numbers were higher (15.11 %) in the clay soils than in the sandy ones (6.02 %) (Table 3.2). Among the sequences that could be classified, the two most dominant classes were *Alphaproteobacteria* and *Betaproteobacteria* (respectively 62.5% and 16.2% on average), being the *Betaproteobacteria* class more abundant in the clay soils (29.5%) than in the sandy ones (3.0%). The other classes that were found varied enormously depending on the type of soil that was analyzed (see Table 3.2). Sequences affiliated with *nif*H genes of *Azospirillum* and *Bradyrhizobium* were found in all eight soils, whereas *Rhizobium nif*H-related sequences were found only in two of the eight soils (Tables 3.2 and S1).



B Soil	B	V	D	W	K	S	G	L
Total sequences	64	80	80	84	87	81	85	81
Observed OTUs	51	223	113	59	111	217	65	66
Chao 1 upper 95%	117	528	213	95	232	381	107	146
Chao 1 lower 95%	32	112	73	46	68	140	50	42

Figure 3.4. Rarefaction curves of observed operational taxonomic units (OTU) based on *nif*H sequences retrieved from the eight soils (A) and corresponding Chao1 richness estimates (B). Rarefaction curves, Chao1 indexes with the corresponding confidence limits, and number of OTU's, were determined by DOTUR. (Schloss and Handelsman, 2008). See legend from figure 3.1 for soil names.

No clear trend could be observed between soil texture and the diversity of nitrogen fixers based on both rarefaction and the Chao1 estimator of richness (Fig. 3.4A and B). However Shannon diversity index based on the same data set revealed a significant higher diversity in the clay (H' = 2.98) than in the sandy (H' = 2.43) soils. Results from UniFrac analysis revealed that the composition of the *nif*H containing community in the four sandy soils did not differ significantly

Table 3.2. Distribution of percentages of *nif*H groups observed in the soil clone libraries.

Genus	Sandy (%)					Clay (%)				
	V*	B*	W*	D*	Mean**	K*	S*	G*	L*	Mean**
Agrobacterium	Nd	Nd	Nd	Nd		1.11	Nd	1.14	3.33	1.40
Azohydromonas	1.11	Nd	1.14	Nd	0.56	Nd	2.35	Nd	1.11	0.87
Azospira	2.22	Nd	Nd	Nd	0.56	1.11	Nd	Nd	1.11	0.56
Azospirillum	3.33	13.85	3.21	8.00	7.10	5.56	8.24	13.64	8.89	9.08
Bradyrhizobium	80.00	6.15	37.50	13.48	34.28	33.33	14.12	51.14	28.89	31.87
Burkholderia	Nd	1.11	3.41	Nd	1.13	Nd	Nd	1.14	Nd	0.28
Clostridium	Nd	21.54	Nd	Nd	5.38	Nd	Nd	Nd	Nd	
Dechloromonas	Nd	Nd	Nd	Nd		13.33	20.00	1.14	6.67	10.28
Derxia	Nd	Nd	Nd	Nd		Nd	1.18	2.27	Nd	0.86
Desulfovibrio	Nd	Nd	Nd	Nd		Nd	1.18	1.14	Nd	0.58
Gluconacetobacter	Nd	18.00	3.52	2.25	5.94	Nd	1.18	Nd	Nd	0.30
Herbaspirillum	Nd	3.08	2.24	Nd	1.33	Nd	Nd	Nd	Nd	
Ideonella	Nd	Nd	Nd	Nd		2.22	1.18	1.14	1.11	1.41
Leptothris	Nd	Nd	Nd	Nd		6.67	Nd	2.27	1.11	2.51
Magnetospirillum	Nd	Nd	1.14	3.37	1.13	Nd	Nd	1.14	Nd	0.28
Mesorhizobium	Nd	1.54	9.09	11.24	5.47	Nd	2.35	2.27	2.22	1.71
Methylobacterium	Nd	Nd	7.95	5.62	3.39	Nd	Nd	1.14	Nd	0.28
Methylocella	1.11	Nd	Nd	2.25	0.84	Nd	Nd	Nd	Nd	
Methylocystis	3.33	Nd	Nd	2.25	1.40	Nd	11.76	Nd	Nd	2.94
Paenibacilus	Nd	23.21	11.36	1.12	8.92	Nd	Nd	Nd	Nd	
Polaromonas	Nd	Nd	Nd	Nd		22.22	8.24	5.68	13.33	12.37
Rhizobium	Nd	Nd	Nd	38.20	9.55	Nd	Nd	Nd	20.00	5.00
Rhodobacter	Nd	1.54	1.14	Nd	0.67	Nd	Nd	Nd	Nd	
Rhodopseudomonas	1.11	Nd	3.41	4.49	2.25	Nd	1.18	Nd	Nd	0.30
Sideroxidans	Nd	Nd	Nd	Nd		1.11	2.35	Nd	Nd	0.87
Sinorhizobium	Nd	1.54	Nd	1.12	0.67	1.11	Nd	Nd	Nd	0.28
Xanthobacter	Nd	Nd	13.64	Nd	3.41	1.11	2.35	Nd	Nd	0.87
Unclassified	7.78	8.45	1.26	6.61	6.02	11.11	22.34	14.77	12.22	15.11

* Soils used in this work: V, Valthermond; B, Buinen; W, Wildekamp; D, Droevendaal; K, Kollummerwaard; S, Steenharst; G, Grebbedijk, L, Lelystad.

** Average percentage in the four sandy and four clay soils.

Nd: not detected

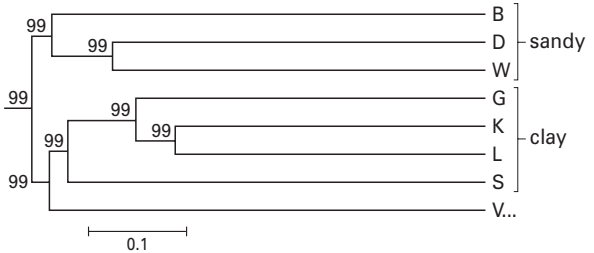


Figure 3.5. Dendrogram based on *nif*H clone libraries, showing the differences in the community structure from eight soil. Distance matrices generated with UniFrac were used to cluster the soils using UPGMA; and jackknife analysis was used to evaluate how robust each environment cluster is to sample size and evenness. Numbers indicate the frequency with which nodes were supported by jackknife analysis. See legend from figure 3.1 for soil names.

among each other, nor did the community in the clay soils (P = 0.06). However, when all environments were included in one comparison, the differences between sequences composition in sandy versus clay soils were significant (P < 0.01, after Bonferroni correction). This was confirmed by jackknife analysis of the clustering (Fig. 3.5).

Influence of physico-chemical and biological soil characteristics on the N-fixing community

Stepwise multiple regressions were performed to identify factors that could explain the observed variation in gene abundance (qPCR) and Shannon diversity index (clone library). Both were normally distributed (Shapiro-Wilk test: P = 0.53 and P = 0.83, respectively). The best predictor (P < 0.05) for the abundance of the N-fixing community for all eight soils during the whole vegetation period was soil pH, explaining 20.7% of the variation (Table 3.3). The remaining variation in the abundance of the N-fixing community over all soils was best explained by DOC (P < 0.05). Regarding the sandy soils, the variation in abundance of N-fixing community was best explained by pH and OM content. In the case of clay soils, the level of NH_4 was the main predictor of *nif*H gene abundance (Table 3.3). The variation observed in *nif*H gene diversity was best predicted by NH4 and DOC, with a negative (P < 0.05) influence of NH_4 that explained 66.5% of the variation.

Discussion

Considering the importance of nitrogen fixation for nitrogen cycling and crop production, we focused our study on the soil diazotrophic communities, which

were explored in respect of their natural fluctuations across eight selected Dutch soils, throughout a growing season. We used direct molecular methods, based on soil DNA, focusing on the *nif*H gene. In order to target the *nif*H gene, we used sets of primers, based on those developed by Poly *et al.* (2001a), that amplified fragments of different size. More specifically, the primer sets used for quantitative PCR amplified a longer fragment (450bp), whereas the fragment amplified for cloning and DGGE was shorter (320bp), the sequence of the latter being nested in the former, what reduces potential pitfalls when comparing the data.

Variations in the abundance of diazotrophic communities

Clay fractions in soils are important in imparting specific physical properties, forming micro- and macroaggregates (Gupta and Roper 2010), and providing microaerophilic or anaerobic conditions that are propitious to nitrogen fixation. This knowledge is consistent with our findings, which showed higher abundances of *nif*H genes in the clay soils compared to the sandy ones. Indeed, it has been shown that 70% of the free-living nitrogen-fixing bacteria are located in the clay fraction (Chotte *et al.*, 2002). Alternatively, the higher *nif*H gene abundance in the clay soils could be explained by the higher pH of these soils, which has been shown to favor the potential for biological nitrogen fixation (Roper and Smith, 1991; Nelson and Mele, 2006). The results from our multiple regression analysis supported this latter conclusion, as soil pH was positively correlated with overall *nif*H gene abundance at all four sampling times. Interestingly, the effect of soil pH on the abundance of diazotrophs varied across the season, being stronger in June, when it explained almost half of the variation in *nif*H gene copy numbers. Availability of carbon is another factor that affects the level of nitrogen fixation in soils, the carbon becoming available from rhizodeposition or via retention of crop residues (Gupta and Roper 2010). Indeed, in our work soil DOC had an overall positive effect on *nif*H gene abundance, albeit to a lesser extent than pH. Similarly, Morales *et al.* (2010) found that the abundance of *nif*H genes was positively correlated with organic carbon levels in agricultural soils.

It is important to realize that the primers used in this study are degenerated, and thus represent a mixture of primers with different binding affinities for different templates. Although degeneracy was necessary, as it allows for greater coverage of genes that are highly polymorphic, amplified DNA can only reflect quantitative abundance of species if the amplification efficiencies are the same for all molecules (von Wintzingerode *et al.*, 1997), and degeneracy may influence the formation of primer-template hybrids. Thus, although it is possible that our approach underestimates the actual gene copy numbers, it gives an indication of the size of the *nif*H harboring communities. Appropriate controls to assess this bias are rarely done due to feasibility and costs in environmental studies, but it is an important concern regarding the validity of the qPCR results

In summary, our results showed that the abundance of diazotrophic bacteria was highly correlated with soil pH, the more neutral pH being most favorable for *nif*H gene abundance. Moreover, it appeared that soil texture was important. As the concentration of clay in soil can be correlated with nitrogenase activity, we hypothesized that this is due to a higher abundance of nitrogen- fixing bacteria (Roper and Smith, 1991). However, to test whether nitrogen fixation rates correlate with *nif*H-containing community size will require further analyses, as we did not measure nitrogenase activities in our soils. Future microcosm experiments, aimed to establish the level of correlation between *nif*H gene abundance and potential nitrogen fixation and to explore the effect of soil type (either due to pH or texture), will provide more concrete evidence.

Structure of nitrogen-fixing communities

Analysis of the nitrogen-fixing communities in the eight soils revealed diverse and dynamic diazotrophic assemblages. Soil type had a greater effect in the beginning than later on in the growing season. It has been shown that agricultural practices, such as fertilization and ploughing, play a major role as determinants of bacterial community structure in soil (Patra *et al.*, 2006; Salles *et al.*, 2006; Wakelin *et al.*, 2009) and these might explain the stronger cluster in the beginning of the growing season.

Using a conceptual framework proposed by Marzoratti *et al.* (2008), we explored possible ecological interpretations based on the structure of diazotrophic communities as determined by DGGE. We could observed that the carrying capacity of the soils was significantly higher in the beginning of the season, as determined by the range-weighted richness (Marzoratti *et al.*, 2008), indicating that the environmental conditions at this period were more favorable to diazotrophic communities than at the end of the year. Despite the changes in richness observed over time, the functional organization of the community, as analyzed by Pareto-Lorenz curves, showed small seasonal variation indicating communities that were highly structured over time (Marzoratti *et al.* 2008). Reduction in richness towards the end of the growing season and the increased in community size indicates that these communities (November) are dominated by diazotrophic species which are better adapted to the higher concentrations of NH_4, regardless of soil type.

Regarding the use of DGGE-based approaches, the number and intensity of bands in a gel do not necessarily give an accurate picture of the microbial community due to the fact that one organism may produce more than one band. Because of these drawbacks, the parameters calculated from DGGE fingerprints should be interpreted as indications and not absolute measurements of microbial community structure and diversity. Nevertheless, the ecological parameters used to analyzed diazotrophic community structure in this study were able to provide a better characterization of the N-fixing community in our soils.

Diversity of nitrogen-fixing communities

In order to identify and characterize dominant *nif*H types, we selected soil samples from June, when significant correlations had been found based on *nif*H gene abundance, to determine the diversity of diazotrophic communities across the eight soils. The sequences recovered from the clone libraries were affiliated with several major groups of bacteria. Not surprisingly, the class *Alphaproteobacteria* was the most dominant class found in our soils, as has been found before (Smit *et al.*, 2001; Buckley and Schmidt, 2003). In our case, it was mostly represented by the order *Rhizobiales*, in particular the genus *Bradyrhizobium*. UniFrac analyses showed two clear clusters formed by sand- and clay-derived sequences, confirming the results obtained by PCA analysis of DGGE patterns. Moreover, the soils under study differed greatly in terms of diversity, higher in clay than in sandy soils, and which could be largely explained by the level of NH_4^+. The negative effect of ammonia on nitrogen fixation has been known for a long time, as the expression of nitrogenase is very often inhibited by the presence of NH_4^+ (Brotonegro, 1974; Houwaard, 1978; Christiansen-Weninger and van Veen, 1991). The decrease in *nif*H gene diversity could thus be explained by assuming the selection of less sensitive types under ammonia pressure. A mention previously, the differential clustering of the soils by texture (sand and clay) could be explained not just by assuming a role for texture, but, alternatively, by invoking one for pH. Additional experiments are however needed to disentangle the effects of texture and pH on diazotrophic communities.

Normal operating range of diazotrophic communities

A high turnover in community composition was observed for nitrogen fixing communities, varying from 60 to 65% throughout the season. This internal structuring of the highly dynamic diazotrophic community indicated that at each season, different species came to significant dominance. Furthermore, the structure and size of diazotrophic communities differed greatly between clay and sandy soils, the former displaying higher abundances and diversity of *nif*H genes across a range of Dutch soils. Moreover, clay soil showed higher amplitude of variation in community size, which indicates that the communities associated with this soil type might be more sensitive to fluctuations associated with the season and agricultural practices. Thus, diazotrophic communities associated with sandy and clay soil were shown to have distinct normal operating ranges. Further investigation is necessary to unravel to what extend the amplitude of variation in community size and structure observed for clay soils affect the functioning of these communities and their ability to cope with environmental stresses.

In conclusion, our results indicate a highly dynamic diazotrophic community, whose structure, size and diversity were mainly driven by soil type, pH and

NH_4^+, respectively. Although these parameters were known to affect nitrogen fixing communities, our analyses allowed us to quantify their relevance for different components of diazotrophic communities across both a temporal and spatial scale.

Acknowledgments

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Table S.1. Distribution of percentages of nifH sequences of some species found in the soil clone libraries.

Species	Sandy %					Clay %				
	V*	B*	W*	D*	Mean **	K*	S*	G*	L*	Mean **
Azospirillum brasilense AWB5	Nd	3.12	1.19	1.25	1.39	Nd	Nd	Nd	Nd	
Azospirillum canadense LMG 23617	1.25	4.68	2.38	Nd	2.07	Nd	Nd	Nd	Nd	
Azospirillum sp. TSA20c	Nd	Nd	Nd	Nd		11.49	Nd	8.23	7.40	6.78
Bradyrhizobium sp. IRBG 228	Nd	6.25	9.52	Nd	3.94	11.49	Nd	22.35	Nd	8.46
Bradyrhizobium japonicum clone 1-18	66.25	Nd	1.19	2.50	17.48	4.59	Nd	1.17	4.93	2.67
Bradyrhizobium japonicum CCBAU 43129	1.25	Nd	19.04	2.50	5.69	Nd	6.17	Nd	Nd	1.54
Bradyrhizobium sp. BTAi1	Nd	1.25	7.14	Nd	2.09	Nd	2.46	8.23	0	2.67
Bradyrhizobium sp. TSA1	Nd	Nd	Nd	Nd		6.89	2.46	2.35	4.93	4.15
Dechloromonas aromatica	Nd	Nd	Nd	Nd		13.79	18.51	1.17	6.17	9.91
Gluconacetobacter diazotrophicus	Nd	14.28	5.95	2.50	5.68	Nd	Nd	Nd	Nd	
Herbaspirillum sp.	Nd	2.50	2.38	Nd	1.22	Nd	Nd	Nd	Nd	
Mesorhizobium loti	Nd	1.25	11.90	1.25	7.20	Nd	2.46	2.35	2.46	1.81
Paenibacillus azotofixans	Nd	Nd	11.90	1.25	6.57	Nd	Nd	Nd	Nd	
Paenibacillus borealis	Nd	14.06	Nd	Nd	7.03	Nd	Nd	Nd	Nd	
Paenibacillus massiliensis	Nd	5.00	Nd	Nd	1.25	Nd	Nd	Nd	Nd	
Polaromonas naphthalenivorans	Nd	Nd	Nd	Nd		21.83	8.64	5.88	14.81	12.79
Rhizobium sp.	Nd	Nd	Nd	40.00	10.00	Nd	Nd	Nd	20.98	5.24
Xanthobacter autotrophicus	Nd	Nd	8.33	Nd	2.08	11.49	2.46	1.17	Nd	3.78

* Soils used in this work: V, Valthermond; B, Buinen; W, Wildekamp; D, Droevendaal; K, Kollummerwaard; S, Steenharst; G, Grebbedijk, L, Lelystad.
** Average percentage in the four sandy and four clay soils. Nd: not detected.

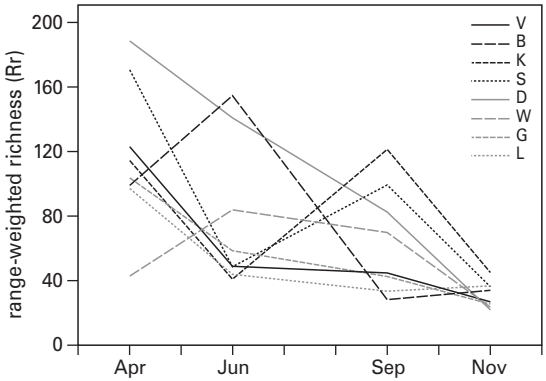

Figure S1. Range-weighted richness (Rr) of the eight soils over time. See legend from figure 3.1 for soil names.

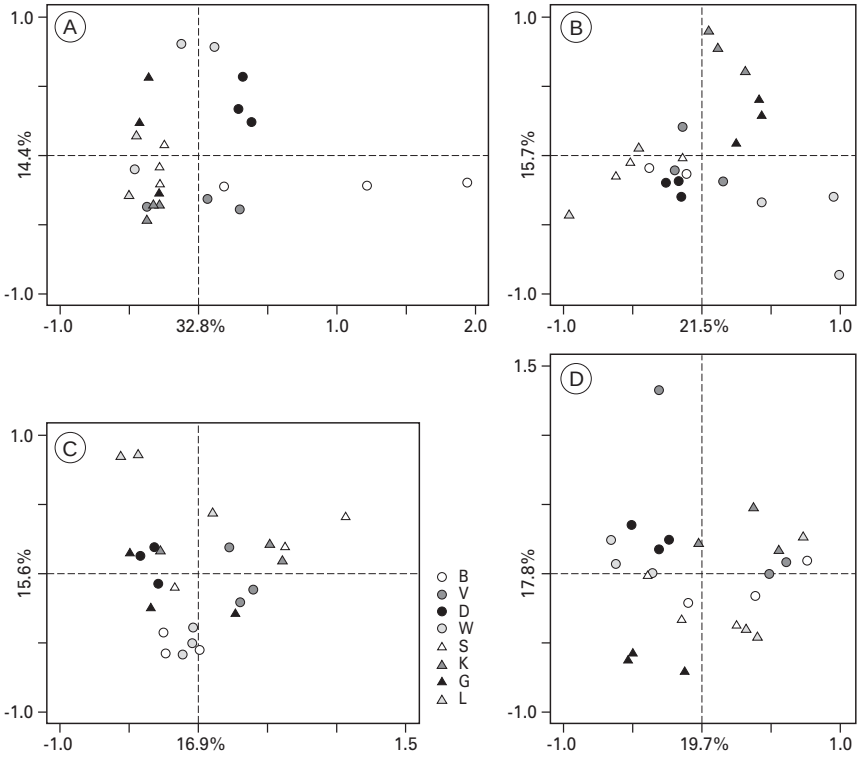

Figure S2. Ordination biplot diagrams generated by principal component analysis (PCA) of N-fixing communities in the eight soils across the four sampling times: April (A), June (B), September (C) and November (D). The eigenvalues on the axes indicate the percent variation explained by PCR-DGGE rybotypes. Symbols represent soil samples; circles correspond to sandy soils, inverted triangles correspond to clay soils. See legend from figure 3.1 for soil names.

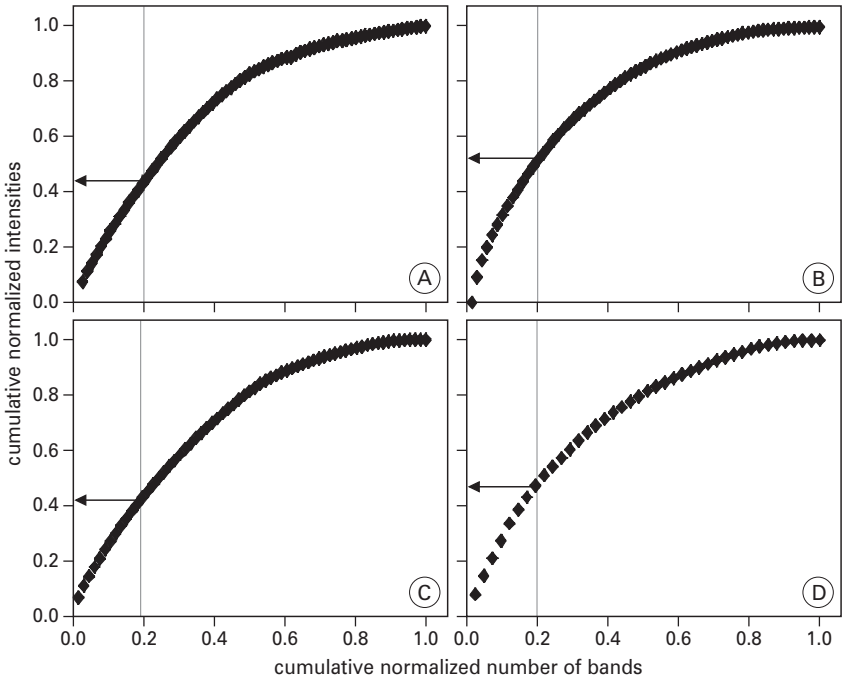

Figure S3. Pareto-Lorenz distribution curves based on DGGE profiles of the N-fixing community over time, based on average values of the sandy and clay soils: April (A), June (B), September (C) and November (D). The dashed vertical line at the *0.2x* axis level is plotted to evaluate the range of the Pareto values.

Chapter 4

Fluctuations in ammonia oxidizing communities across agricultural soils are driven by soil structure and pH

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Abstract

The milieu in soil in which microorganisms dwell is never constant. Conditions such as temperature, water availability, pH and nutrients frequently change, impacting the overall functioning of the soil system. To understand the effects of such factors on soil functioning, proxies (indicators) of soil function are needed that, in a sensitive manner, reveal normal amplitude of variation. Thus, the so-called normal operating range (NOR) of soil can be defined. In this study we determined different components of nitrification by analyzing, in eight agricultural soils, how the community structures and sizes of ammonia oxidizing bacteria and archaea (AOB and AOA, respectively), and their activity, fluctuate over spatial and temporal scales. The results indicated that soil pH and soil type are the main factors that influence the size and structure of the AOA and AOB, as well as their function. The nitrification rates varied between 0.11 ± 0.03 $\mu gN.h^{-1}.gdw^{-1}$ and 1.68 ± 0.11 $\mu gN.h^{-1}.gdw^{-1}$, being higher in soils with higher clay content (1.09 ± 0.12 $\mu gN.h^{-1}.gdw^{-1}$) and lower in soils with lower clay percentages (0.27 ± 0.04 $\mu gN.h^{-1}.gdw^{-1}$). Nitrifying activity was driven by soil pH, mostly related to its effect on AOA but not on AOB abundance. Regarding the influence of soil parameters, clay content was the main soil factor shaping the structure of both the AOA and AOB communities. Overall, the potential nitrifying activities were higher and more variable over time in the clayey than in the sandy soils. Whereas the structure of AOB fluctuated more (62.7 ± 2.10%) the structure of AOA communities showed lower amplitude of variation (53.65 ± 3.37%). Similar trends were observed for the sizes of these communities. The present work represents a first step towards defining a NOR for soil nitrification. The sensitivity of the process and organisms involved to impacts from the milieu support their use as proxies in the NOR of agricultural soils. Moreover, the clear effect of soil texture established here suggests that the NOR should be defined in a soil-type-specific manner.

Introduction

The diversity of microorganisms on Earth is astonishing. Torsvik *et al.* (1990) estimated the number of bacterial genomes in a mixed sample using DNA:DNA hybridization. The number of bacterial species in a gram of boreal forest soil was estimated to approximate 10,000. Recently, this number was reevaluated and estimations are that the number of bacterial types per gram of soil varies between 2,000 in polluted soil and 8.3 million in pristine soil (Gans *et al.*, 2005; Schloss and Handelsman, 2006). Microorganisms dominate soil communities and have a profound impact on ecosystem functioning, being drivers of key processes in the cycling of energy and nutrients. The environment in which these microorganisms dwell is, however, never constant. Abiotic and biotic conditions often change with time, leading to fluctuations in the soil microbial communities and in the overall functioning of the soil ecosystem. The natural variation of soil processes over time, in response to fluctuations in ecological factors can be depicted as the highs and lows in soil process rates. These high and lows comprise the upper and lower borders of what has been coined the normal operating range (NOR). The NOR thus represents the amplitude of variation of a given process/parameter under natural (field) conditions, over time. The manner in which the NOR is defined will depend on the spatial and temporal scales at which measurements are taken. For instance, the accumulation of organic matter (OM) in a forest is a slow process. Thus, in order to capture the natural amplitude of variation in OM in forest soils, measurement should be taken over a large timespan, on the order of decades. Local conditions are also likely to influence OM deposition, and therefore, measurements may be taken in similar forests across a region of interest. For processes that respond fast to changes caused by weather and/or anthropogenic activities, such as nitrification, a NOR might be defined on the basis of a shorter (one to a few year) study. Thus, due to its sensitivity to external drivers, processes like nitrification are considered to represent good indicators of soil quality (Doran and Zeiss 2000; Bruinsma et al, 2003).

The NOR of soil functioning is of key relevance when evaluating the impact of disturbances on soil-borne ecosystem services and processes. Examples of such potential soil perturbations are extreme abiotic events (drought, flooding, fire), changes in agricultural management or land use, and/or the planting of genetically-modified (GM) crops. Defining a NOR is important, as it provides a background against which to compare the extent of the effects of such, and other, disturbances (van Straalen, 2002; Kowalchuk *et al.* 2003; Bruinsma *et al.* 2003). For instance, by determining the fluctuations in the bacterial diversity associated with a suite of potato plants, Inceoglu *et al.* (2011) showed that the physiological changes associated with a GM potato did not affect the bacterial community in its rhizosphere differently from the effects of five other cultivars.

Moreover, by incorporating perturbance-sensitive processes (and their proxies) into a mathematical model, an overall NOR of soil may be determined, which may be of use as a parameter that indicates the overall soil quality (Pereira e Silva *et al.*, submitted). This overall NOR will represent a statistical tool that provides a score for soil functioning. Once the NOR is defined, it can be used to detect statistically significant changes in soil functioning, in response to disturbances at a specific time point.

Agricultural systems annually receive approximately 25% of global nitrogen input, mostly in the form of ammonium (Gruber and Galloway, 2008). The added ammonium can be oxidized to nitrate in a two-step process called nitrification. Besides its ecological relevance, nitrification is considered to represent a perturbation-sensitive process, and as such it has been advocated as a potentially suitable indicator of soil quality, e.g. in the risk assessment of GM plants (Bruinsma *et al.* 2003; Kowalchuk *et al.* 2003; Wessen and Hallin, 2011; Ritz *et al.*, 2009). The oxidation of ammonia, the first (and rate-limiting) step in the nitrification process (performed by ammonia monooxygenase, which is encoded by amo genes), until recently was considered to be largely performed by just two monophyletic groups within the gamma- and beta-proteobacteria (AOB). AOB have been frequently used as indicators of perturbations, to measure the effects of pollution in fish farm sediments (McCaig *et al.* 1999), contamination of soil with toxic metals (Stephen *et al.*, 1999), effect of effluent irrigation (Oved *et al.*, 2001) and organic waste residues (Horz *et al.*, 2004; Nyberg *et al.*, 2006). However, ammonia oxidizing archaea belonging to the recently described thaumarchaea (AOA; Spang *et al.*, 2010) have been identified several years ago (Schleper *et al.*, 2005; Treusch *et al.*, 2005) and these organisms were found to respond to environmental factors (Ying *et al.* 2010). They often revealed a remarkable numerical dominance in soils (Leininger *et al.* 2006).

Both AOA and AOB play roles in nitrification, although the exact contribution of each one of the two communities to the process remains unclear. There is evidence that ammonia oxidation by archaea may exceed that performed by bacteria in some soils (Offre *et al.*, 2009; Prosser and Nicol, 2008; Tourna *et al.*, 2008). In contrast, Jia and Conrad (2009) found that, after ammonium addition, the changes in nitrification activity were paralleled by changes in the abundances of AOB but not of AOA. Thus, the likely involvement of the AOA in the process (Wessen *et al.* 2011; Yao *et al.* 2011; Zhang *et al.* 2009; Caffrey *et al.* 2007) suggested that AOA, in conjunction with AOB, should be used as proxies to monitor nitrification. Accordingly, both AOA and AOB have been recently suggested as good indicators of soil quality (Wessen and Hallin, 2011).

Considering the great importance of nitrification and the usefulness of nitrifiers as bioindicators of soil quality, the aim of this work was to determine the NOR of nitrification across agricultural soils. For that purpose, we assessed nitrification across eight soils over two years. The NOR that was thus obtained

represents a descriptive measure which illustrates the amplitude of variation of nitrification and/or its proxies under prevailing conditions in the soils, over eight locations and time. In particular, we determined the size, structure and diversity of both AOA and AOB communities across the soils. Community sizes were studied by quantifying the archaeal and bacterial *amo*A genes, whereas community structures were determined by PCR-DGGE of archaeal *amo*A and (betaproteo)bacterial 16S rRNA genes. Moreover, we constructed bacterial and archaeal *amo*A clone libraries to identify the dominant types. Finally, we also measured relevant chemical soil parameters. We hypothesized that the ammonia oxidizing communities would be mainly driven by soil type and pH, suggesting that a NOR should be defined per soil (textural) type.

Material and Methods

Experimental sites and soil sampling

Eight soils from different sites in the Netherlands were sampled seven times between April 2009 and October 2010, after seedling (April 2009 and 2010), before flowering (June 2009 and 2010), and in senescence stage (September 2009 and October 2010). In November 2009 there were no plants in the fields anymore. The fields are used for potato cropping and were under agricultural rotation regime. Information on land-use and location is available (Table 4.1). The soils were chosen to represent different soil types (clay vs. sand) and present different chemical properties (Table 4.2). Bulk soil samples (4 replicates per soil; 0.5kg per replicate) were collected in plastic bags and thoroughly homogenized before further processing in the lab. A 100-g subsample was used for measuring ammonia oxidizing enzyme activity, molecular biology and soil chemical properties.

Table 4.1. Specific data for each soil concerning soil type, land use as well as GPS coordinates.

Sampling Site	Sand:Silt:Clay (%)	Soil type	Land use	North coordinate	East coordinate
Buinen (B)	50:20:30	Sandy loam	Agricultural	52°55′386″	006°49′217″
Valthermond (V)	55:40:5	Sandy loam	Agricultural	52°50′535″	006°55′239″
Droevendaal (D)	55:20:25	Sandy loam	Agricultural	51°59′551″	005°39′608″
Wildekamp (K)	50:25:25	Sandy loam	Natural grassland	51°59′771″	005°40′157″
Kollumerwaard (K)	20:50:30	Clayey	Agricultural	53°19′507″	006°16′351″
Steenharst (S)	30:20:50	Silt loam	Agricultural	53°15′428″	006°10′189″
Grebedijk (G)	8:12:80	Clayey	Agricultural	51°57′349″	005°38′086″
Lelystad (L)	8:12:80	Clayey	Agricultural	52°32′349″	005°33′601″

Table 4.2. Soil characteristics measured in this study.

Soil location	pH							OM (%)							Water content (%)						
	A[1]	J[1]	S[1]	N[1]	A[2]	J[2]	O[2]	A[1]	J[1]	S[1]	N[1]	A[2]	J[2]	O[2]	A[1]	J[1]	S[1]	N[1]	A[2]	J[2]	O[2]
Buinen (B)	ND	4.4	4.3	4.4	4.2	4.6	4.4	ND	3.6	3.6	4.1	4.9	3.5	3.6	11.6	9.8	4.6	16.2	13.8	7.5	12.3
Valthermond (V)	4.4	4.6	4.8	5.1	4.3	4.5	4.8	12.9	14.2	20.0	24.6	15.8	15.7	19.8	45.7	30.2	30.8	48.9	25.1	34.3	55.0
Droevendaal (D)	5.0	5.3	5.1	5.5	5.0	5.1	4.7	2.8	3.4	2.9	3.6	2.6	3.3	2.6	15.1	14.5	7.7	17.3	9.5	16.0	9.4
Wildekamp (W)	ND	4.6	4.6	5.0	4.7	4.7	4.7	ND	3.3	3.7	6.8	4.6	2.7	3.6	14.4	19.8	6.9	19.3	11.5	22.7	10.2
Kollumerward (K)	ND	7.5	7.6	7.5	7.4	7.4	7.4	ND	2.7	2.6	3.3	6.6	3.3	2.7	19.3	19.6	14.6	23.6	16.7	19.7	21.5
Steenharst (S)	5.7	5.7	5.7	5.5	5.1	5.4	5.4	5.9	5.6	4.0	6.4	6.5	5.0	4.4	32.9	31.0	9.3	22.8	41.2	35.7	25.3
Grebbedijk (G)	ND	7.5	7.5	7.6	7.2	7.0	7.4	ND	4.8	5.4	5.6	6.4	4.3	5.5	23.6	21.9	14.1	22.0	18.8	20.4	19.6
Lelystad (L)	7.5	7.4	7.5	7.7	7.2	7.4	7.4	2.7	2.6	2.8	4.3	2.8	2.5	3.2	17.7	12.6	10.0	19.0	13.4	9.2	17.2
Average	5.6	5.9	5.9	6.0	5.6	5.8	5.5	6.1	5.0	5.6	7.3	6.3	5.0	5.7	22.5	19.9	12.3	23.6	18.8	20.7	21.3
Sd deviation	1.3	1.4	1.4	1.3	1.4	1.3	1.2	4.8	3.9	5.9	7.1	4.2	4.4	5.8	11.5	7.7	8.2	10.5	10.3	10.3	14.7
Sd error	0.4	0.28	0.3	0.3	0.3	0.3	0.3	1.4	0.8	1.2	1.5	0.9	0.9	1.2	2.3	1.6	1.7	2.2	2.1	2.1	3.0

A = April; J = June; S = September ; O = October and N = November. 1 year 2009 and [2], year 2010. In April only four soils were analyzed and therefore this time was excluded from further analysis. OM = organic matter; $N\text{-}NO_3^-$ = nitrate and $N\text{-}NH_4^+$ = ammonium. Numbers are average of three replicates. ND, not determined.

Soil chemical analysis and ammonia oxidizing activity

Soil pH was defined in 0.01M $CaCl_2$ (1:4.5). Water content was determined by drying for 48h at 65ºC. Organic matter (OM) content was calculated on dried soil as the difference between the initial and final sample weights measured after 4 hours at 550ºC. Nitrate ($N\text{-}NO_3^-$) and ammonium ($N\text{-}NH_4^+$) were determined colorimetrically in a solution of 0.01 M $CaCl_2$ with an Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, New York) (samples from 2009) and using the commercial kits Nanocolor Nitrat50 (detection limit, 0.3 mg N kg^{-1} dry weight, Macherey-Nagel, Germany) and Ammonium3 (detection limit, 0.04 mg N kg^{-1} dry weight; Macherey-Nagel, Germany) (samples from 2010) according to Töwe *et al.* (2010) . Potential nitrifying enzyme activity (NEA) was measured in soil suspensions in the presence of non-limiting ammonium and ambient atmospheric O_2 concentration according to Dassonville *et al.* (2011), using a modified version of the method of Hart *et al.* (1994) with an ionic chromatography (DX120, Dionex, Salt Lake City, USA) equipped with a 4 × 250 mm column (IonPac AS9 HC).

Nucleic acid extraction

DNA was extracted from 0.5 g of soil using Power Soil MoBio kit (Mo Bio Laboratories Inc., NY), according to the manufacturer's instructions, after the addition of glass beads (diameter 0.1 mm; 0.25 g) to the soil slurries. The cells were disrupted by bead beating (mini-bead beater; BioSpec Products, United States) three times for 60 s. The quantity of extracted DNA was estimated by comparison to a 1-kb DNA ladder (Promega, Leiden, Netherlands) and quality was determined based on the degree of DNA shearing (average molecular size) as well as the amounts of coextracted compounds.

Real-time quantitative PCR

The abundance of archaeal and bacterial ammonia oxidizers was quantified by quantitative PCR (qPCR) targeting the *amo*A gene. For AOA primers amo23F (Tourna *et al.*, 2008) and crenamo616r (Nicol *et al.*, 2008) were used obtaining fragments of 624 bp. AOB *amo*A quantification was performed using primers amoA-1F (Stephen *et al.*, 1999) and amoA-2R (Rotthauwe *et al.*, 1997), according to Nicol *et al.* (2008), generating fragments of 491 bp. Cycling programs and primer sequences are detailed in Table 4.3. Quantification was carried out twice from each of the four soil replicates on the ABI Prism 7300 Cycler (Applied Biosystems, Germany). The specificity of the amplification products was confirmed by melting-curve analysis, and the expected sizes of the amplified fragments were checked in a 1.5% agarose gel stained with ethidium bromide. Standard curves were obtained using serial dilutions of plasmid containing cloned archaeal or bacterial *amo*A gene, from 10^7 to 10^2 gene copy numbers/µl. Possible inhibitory effects of co-extracted humid compounds were checked by

spiking standard concentrations with samples. No apparent inhibition was observed.

Standard PCR amplification and DGGE analysis

PCR was performed targeting 16S rRNA and *amo*A genes of ammonia-oxidizing bacteria (AOB) or ammonia-oxidizing archaea (AOA), respectively. Amplification of 16S rRNA gene fragments from extracted soil DNA was achieved by primary amplification with CTO189f and CTO654r primers (Kowalchuk *et al.*, 1997) and with a secondary nested amplification using bacterial 357f-GC and 518r primers (Muyzer *et al.*, 1993). CTO and bacterial primers amplified 465 and 161bp fragments, respectively. A detailed procotol is described in Freitag *et al.* (2006). Ammonia oxidizing archaea *amo*A was amplified using primers crenamA23f/crenamoA616r (Tourna *et al.*, 2008). Cycling conditions are described in Table 4.3. DGGE profiles were generated with the Ingeny Phor-U system (Ingeny International, Goes, The Netherlands). The PCR products (120 ng/ lane) were loaded onto 6% (w/v) polyacrylamide gels, with a 15–55% and 35–70% denaturant gradient (100% denaturant corresponded to 7 M urea and 40% (v/v) deionized formamide) for archaeal *amo*A and 16S rRNA gene, respectively, as described previously by Nicol *et al.* (2008). Electrophoresis was performed at a constant voltage of 100 V for 16 h at 60ºC. The gels were stained for 60 min in 0,5× TAE buffer with SYBR Gold (final concentration 0,5 µg/liter; Invitrogen, Breda, The Netherlands). Images of the gels were obtained with Imagemaster VDS (Amersham Biosciences, Buckinghamshire, United Kingdom) and normalized in the GelCompar II software (Applied Maths, Sint-Martens Latem, Belgium), using the unweighted-pair group method with arithmetic mean, rolling-disk background subtraction, and no optimization (Kropf *et al.*, 2004; Rademaker, *et al.*, 1999). Patterns were compared by clustering the different lanes by Pearson's correlation coefficient implemented in GelCompar.

Construction of AOA and AOB libraries and phylogenetic analysis

Clones libraries of archaeal and bacterial *amo*A genes for the eight soils were constructed using DNA extracted from soil collected in June of 2010. Primers Crenam23f /Crenamo616r for archaeal *amo*A, and primers amoA-1R/ amoA-2R for bacterial *amo*A were used, as described for real time PCR. The products from replicates were pooled per soil, ligated into PGEM-T-Easy vector (Promega, Madison, WI, EUA) in accordance with the manufacturer's instructions and white colonies were subject to a colony PCR with vector specific primers M13-F and M13-R to check for the presence of *amo*A inserts. DNA sequencing was performed using an Applied Biosystems 3730 XL DNA Analyzer at LGC Genomics GmbH (Berlim, Germany). Short sequences or sequences of chimeric origin were checked by analyzing alignments using Bellerophon (Huber *et al.*, 2004) and excluded from the analysis. Sequences obtained were processed in Mega

(version 5, Mega, Biodesign Institute), translated, and the deduced amino acid sequences were aligned using Clustal W (Jeanmougin, 1998). Sequences at 1% cut-off were used to construct phylogenetic trees in which representative sequences from GenBank were included. Distance analysis of derived archaeal and bacterial *amo*A protein sequences and bootstrap support (neighbor-joining and parsimony analysis; 1000 replicates each) were constructed in Mega, using Jones-Taylor-Thornton (JTT) substitution model with site variation (invariable

Table 4.3. PCR and cycling conditions for PCR-DGGE analysis and real time quantification of AOA and AOB genes.

	Primer sequence (5′- 3′)	Thermal conditions
Primers DGGE		
***amo*A (AOA):**		
CrenamoA23f CrenamoA616r (Tourna *et al.*, 2008)	ATGGTCTGGCTWAGACG GCCATCCATCTGTATGTCCA	95ºC 5 min 94ºC 30 s, 55ºC 30 s, 72ºC 1 min, 10 cycles 92ºC 30 s, 55ºC 30 s and 72ºC 1 min, 25 cycles final ext. of 72ºC 10 min
***amo*A (AOB):**		
CTO189f A CTO189f B CTO189f C CTO654r (Kowalchuk *et al.*, 1997)	GGAGRAAAGCAGGGGATCG GGAGRAAAGCAGGGGATCG GGAGGAAAGTAGGGGATCG CTAGCYTTGTAGTTTCAAACGC	93°C, 60 s 92°C 30 s, 57°C 1 min, 68°C 45 s, 35 cycles final ext. of 68ºC 5 min
P3 (357f-GC) P2 (518r) (Muyzer *et al.*, 1993)	CGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	93°C, 60 s 92°C 30 s, 57°C 1 min, 68°C 45 s, 35 cycles final ext. of 68ºC 5 min
Primers real time PCR		
***amo*A(AOA):**		
amo23F (Tourna *et al.*, 2008) CrenamoA616r48x (Nicol *et al.*, 2008)	ATGGTCTGGCTWAGACG GCCATCCABCKRTANGTCCA	95°C, 10 min, 1 cycle 94°C 45 s, 50°C 45 s, 72°C 45 s, 39 cycles
***amo*A(AOB):**		
amoA-1F (Stephen *et al.*, 1999) amoA-2R (Rotthauwe *et al.*, 1997)	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	95°C for 10 min, 1 cycle 94°C 1 min, 60°C 1 min, 72°C 1 min, 39 cycles

sites and eight variable gamma rates) (Olsen *et al.*, 1994). Differences in the community structures of AOA and AOB clone libraries were analyzed with UniFrac (Lozupone *et al.*, 2006), and the program DOTUR (Distance-based OTU and richness) (Schloss and Handelsman, 2008) was used to create rarefaction curves and to determine the Shannon diversity index.

Data analysis

Physicochemical variables were checked for normality and were log-transformed, except for soil pH. Differences in these variables between sandy and clay soils, among all eight soils, and over time were assessed with Student's T-tests.

To test the influence of soil physicochemical parameters (environmental factors) on community structure, forward selection was used on CCA, to select a combination of environmental variables that explained most of the variation observed in the AOA and AOB species matrix. For that, a series of constrained CCA permutations was performed in Canoco (version 4.0 for Windows, PRI Wageningen, The Netherlands,) to determine which variables best explained the assemblage variation, using automatic forward selection and Monte Carlo permutations tests (permutations = 999).The length of the corresponding arrows indicated the relative importance of the chemical factor explaining variation in the two microbial communities.

To study the dynamics of AOA and AOB communities over time, a matrix of similarities based on Pearson's correlation was used to perform moving window analysis - MWA (Marzeroti *et al.*, 2008), by calculating the rate of change in community structure, as *dissimilarity = 100 – similarity%* (represented by Δt).The rate of change parameter (Δt) averages the degree of change between consecutive DGGE profiles of the same community over a fixed time interval (Marzorati *et al.*, 2008), giving an indication of community turnover for that time period.

Correlations between NEA and community structure were tested using the RELATE analysis, a non-parametric form of Mantel test, implemented in PRIMER-E software package (version 6, PRIMER-E Ltd, Plymouth, UK; Clarke and Gorley, 2006). More specifically, for each functional group (AOA or AOB), a rank correlation coefficient (here Spearman coefficient) and significance level (obtained by a permutation test using 5000 permutations) were computed to quantify the correlation between the rank similarity matrices obtained for activity and genetic structure (Clarke and Ainsworth, 1993). For each functional group, ANOSIM statistics (Primer-E software) was performed to test for an effect of soil type on AOA and AOB community structure.

The NOR of NEA and the abundance of ammonia oxidizers were determined by subtracting lower values from higher values (also represented by Δt) of activity or *amo*A gene copy numbers, respectively. The influence of soil parameters or abundance of ammonia oxidizers on NEA was determined by using Pearson's

linear correlation coefficient implemented in SPSS 16.0 (SPSS, Inc, IL).

We also conducted multiple regression analyses on log-normalized data (SAS® system for Windows version 8.02, SAS Institute Inc, Cary, NC, USA, 2001) to identify the main drivers of nitrification. The following parameters were included in the analysis: diversity (Shannon diversity index *H'* based on DGGE profiles) and abundance (*A*) of archaeal (AOA) and bacterial (AOB) ammonia oxidizers, potential nitrifying enzyme activity (NEA), nitrate (NO_3), ammonium (NH_4), pH, organic matter in % (OM), clay content in % (clay) and soil moisture in % (humidity).

Data accessibility

The *amo*A sequences for the AOA and AOB have been deposited in the GenBank under accession number JF935450 - JF936076 and JF936077 - JF936667 for AOA and AOB, respectively.

Results

Seasonal variations of soil chemical properties

Soil pH, nitrate, ammonium and organic matter levels and water content were determined in triplicate across all soil samples. Overall, considering all soils, soil pH was significantly higher ($P < 0.05$) in soils K, G and L (7.32 ± 0.06, n = 57) than in soils B, V, D, W and S (4.88 ± 0.04, n = 99) during the whole experimental period and no significant variation over time was observed. Significant changes were observed in levels of nitrate at all times, with lower values in the end of the season (September 2009: 32.8 mg/kg ± 7.08; October 2010: 24.2 mg/kg ± 2.98) and higher at the beginning (April 2009: 75.6 mg/kg ± 12.5; April 2010: 56.4 mg/kg ± 5.63). Levels of ammonium also varied over the whole period, lower values being observed at the end of the season (September 2009: 1.92 mg/kg ± 0.16; October 2010: 5.86 mg/kg ± 0.63), and higher ones at the start (April 2009: 13.3 mg/kg ± 1.13; April 2010: 15.3 mg/kg ± 1.01). Significant fluctuations ($P < 0.05$) in water contents in the soils were detected at all times in 2009, but not 2010. The most humid sampling time was November (23.64% ± 2.15), and the driest was September (12.25% ± 1.68). Variations in organic matter content were observed from September (5.63% ± 1.20) to November (7.34% ± 1.45) 2009, and from April (6.28% ± 0.85) to June (5.04% ± 0.89) 2010. Individual values for each soil at each sampling time can be found in Table 4.2. Concerning differences between sandy and clayey soils, soil pH was significantly higher ($P < 0.05$) in the clayey (6.9 ± 0.17, n = 78) than in the sandy soils (4.71 ± 0.06, n = 78). Levels of ammonium and nitrate were significantly higher ($P < 0.05$) in the sandy soils in June and September of 2009 ($N\text{-}NH_4^+$: 13.6 ± 0.97 and 2.3 ± 0.15 mg/kg, respectively; $N\text{-}NO_3^-$: 103.3 ± 11.20 and 52.4 ± 8.41 mg/kg, respectively),

and no significant difference in the organic matter content was found between sandy and clayey soils at any of the sampling times.

Seasonal variation of nitrifying enzyme activity (NEA) in relation to soil parameters

Variations in NEA over time were observed in all soils. On average per time, lower rates were observed in November 2009 (0.59 $\mu gN.h^{-1}.gdw^{-1} \pm 0.09$) and June 2010 (0.59 $\mu gN.h^{-1}.gdw^{-1} \pm 0.07$), whereas higher rates were detected in April 2010 (0.76 $\mu gN.h^{-1}.gdw^{-1} \pm 0.10$) and October 2010 (0.79 $\mu gN.h^{-1}.gdw^{-1} \pm 0.11$). More specifically, significantly higher values were observed for soils V, K, S, G and L (on average 1.00 $\mu gN.h^{-1}.gdw^{-1} \pm 0.04$, n = 100) compared to soils B, D and W (on average 0.15 $\mu gN.h^{-1}.gdw^{-1} \pm 0.00$, n = 60) (Figure 4.1A). The former ones also showed higher variability across the sampling times. Higher

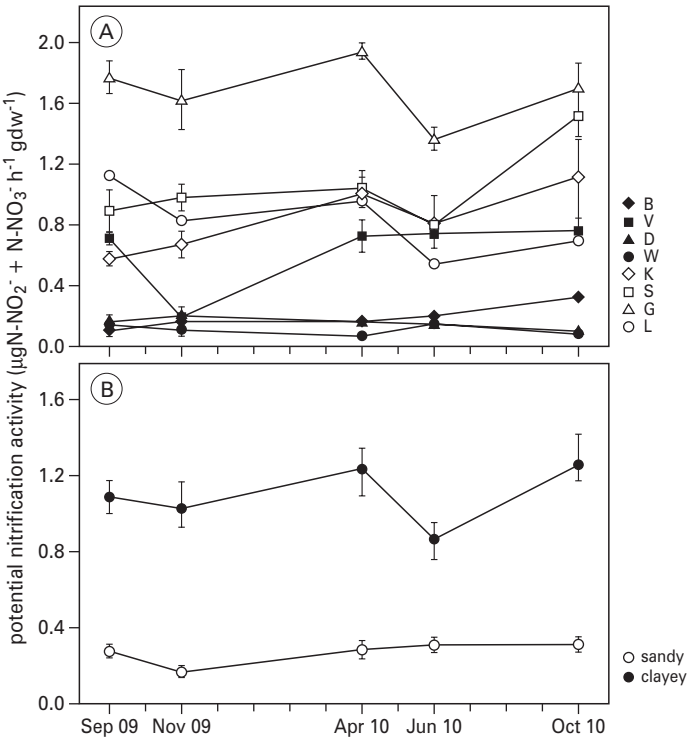

Figure 4.1. Potential nitrifying enzyme activity (NEA) measured in the eight soils from September 2009 to October 2010 (A), and the difference observed in the amplitude of variation in sandy (Δt_{sandy} = 0.14 $\mu gN.h^{-1}.gdw^{-1} \pm 0.04$), and in clayey soils ($\Delta t_{clayey}$ = 0.39 $\mu gN.h^{-1}.gdw^{-1} \pm 0.08$) over time (B), calculated by subtracting lower from higher values. Soil names are: B, Buinen; V, Valthermond; D, Droevendaal; W, Wildekamp; K, Kollumerwaard; S, Steenharst; G, Grebbedijk and L, Lelystad. Bars are standard errors (n = 4).

rates were observed in soil G (1.68 $\mu gN.h^{-1}.gdw^{-1} \pm 0.12$), and lower ones in soil W (0.11 $\mu gN.h^{-1}.gdw^{-1} \pm 0.03$). On average per soil type, clayey soils had significantly higher values than sandy ones (1,055 $\mu gN.h^{-1}.gdw^{-1}$ and 0,261 $\mu gN.h^{-1}.gdw^{-1}$, respectively; $P < 0.05$; Figure 4.1B).

Pearson's correlation analysis between NEA and soil physico-chemical parameters over time revealed that the enzyme activities correlated positively only with soil pH ($r = 0.70$, $P \leq 0.05$) (Table 4.4), but a small yet significant effect of clay content was also identified by multiple regression analysis (Table 4.5). When the same analysis was repeated for sandy and clayey soils separately, we observed that nitrate and organic matter were also important explanatory variables (Table 4.5).

Seasonal variation in the abundance of AOA and AOB communities in relation to soil parameters

Considering all eight soils, both the AOA and AOB abundances varied within 1 to 2 orders of magnitude across the sampling times. The numbers of archaeal *amo*A genes were in the range of 5.94×10^5 to 2.53×10^7 gene copies per gram of dry soil, whereas the bacterial *amo*A gene numbers varied between 2.95×10^5 to 8.32×10^7 gene copies per gram of dry soil. The AOA abundance was significantly higher in June 2010 and lower in April 2009 ($P < 0.05$), whereas the AOB

Table 4.4. Pearson's correlations (r) between community size (qAOA, qAOB and AOA/AOB), nitrifying activity (NEA) and soil properties (OM, $N\text{-}NO_3^-$, $N\text{-}NH_4^+$, pH, moisture and clay), calculated as average values per soil between September 2009 and October 2010.

	OM	$N\text{-}NO_3^-$	$N\text{-}NO_4^+$	pH	Moisture	Clay	qAOA	qAOB	AOA/AOB	NEA
OM	1	NS	NS	NS	*	NS	NS	NS	NS	NS
$N\text{-}NO_3^-$	0.57	1	*	NS	NS	NS	NS	NS	NS	NS
$N\text{-}NO_4^+$	0.55	**0.74***	1	NS	NS	NS	NS	NS	NS	NS
pH	-0.44	-0.58	-0.23	1	NS	*	*	NS	NS	*
Moisture	**0.76***	0.47	0.33	-0.09	1	NS	NS	NS	NS	NS
Clay	-0.12	-0.39	0.25	**0.72***	-0.06	1	NS	NS	NS	NS
qAOA	0.17	-0.04	0.34	**0.73***	-0.13	0.49	1	**	NS	*
qAOB	0.35	0.22	0.24	0.15	0.06	0.04	**0.84****	1	NS	NS
AOA/AOB	0.10	-0.02	0.29	0.05	0.19	0.41	-0.06	-0.46	1	NS
NEA	0.16	-0.28	0.09	**0.70***	-0.15	0.48	**0.74***	0.45	0.41	1

Abreviations: NEA, potential nitrifying enzyme activity; AOA, ammonia oxidizing archaeal; AOB, ammonia oxidizing bacteria; NS, not significant ; *** $P < 0.001$, ** $P < 0.01$; * $0.01 < P < 0.05$. Values in bold are significant.

abundance was the highest in October 2010, with comparable numbers at the other sampling times (Figures 4.2A, B, C). In general, AOA and AOB population sizes tended to increase from April to June in both years, decreasing in November (Figure 4.2A). Abundances of archaeal *amo*A genes varied from 4.76×10^5 to 3.58×10^6 gene copies gdw^{-1} in the sandy soils and from 1.40×10^6 to 1.54×10^7 gene copies gdw-1 in the clayey soils (Figure 4.2B). The bacterial *amo*A gene numbers varied from 1.74×10^6 to 2.30×10^7 gene copies gdw^{-1} in the sandy soils and from 1.60×10^6 to 4.53×10^7 gene copies gdw^{-1} in the clayey soils (Figure 4.2C). The observed amplitude of variation in community size between AOA and AOB was significantly different in April and June 2009 and in October 2010 and was larger for AOB ($\Delta t_{AOB} = 1.35 \pm 0.14$), especially in the sandy soils ($\Delta t_{clay} = 1.45 \pm 0.3$ and $\Delta t_{sandy} = 1.26 \pm 0.08$), than for AOA (_tAOA = 1.21 ± 0.08) abundances, which was higher in the clayey soils ($\Delta t_{clay} = 1.25 \pm 0.02$ and $\Delta t_{sandy} = 1.18 \pm 0.02$) (especially in the clayey soils (Figures 4.2B, C).

Pearson's correlations between the archaeal and bacterial *amo*A gene copy numbers and the soil chemical parameters measured revealed that only soil pH significantly affected the abundance of AOA ($r = 0.73$, $P < 0.05$), but showed no influence on the abundance of AOB (Table 4.4).

Analysis of AOA and AOB community structure and diversity in relation to soil parameters

Two-way analysis of similarities (ANOSIM) showed an overall effect of soil type on AOA and AOB community structures at all times, but to a lesser extent on AOB (Table S1). Based on R values, the greatest community differentiations became measurable during early fall for AOA, but during spring and summer

Table 4.5. Best regression models for NEA in the eight soils over five sampling times.

Soil	Model	P-value	R^2
Overall	$^{a}NEA = -2.35^{**}(\pm0.33) + 2.02^{**}(\pm0.43) \times pH + 0.01^{**}(\pm0.001) \times clay$	<0.0001	0.53
	$^{b}NEA = -1.15^{**}(\pm0.48) + 0.36^{**}(\pm0.07) \times A_{AOA} - 0.19^{*}(\pm0.07) \times H_{AOA}$	<0.0001	0.17
Sandy soils	$^{a}NEA = 0.67(\pm0.41) + 0.21^{**}(\pm0.06) \times NO_3 + 0.35^{**}(\pm0.08) \times OM$	0.0003	0.56
	$^{b}NEA = -1.22^{*}(\pm0.47) + 0.11^{**}(\pm0.05) \times A_{AOA} - 0.17^{**}(\pm0.06) \times H_{AOA}$	<0.0001	0.24
Clayey soils	$^{a}NEA = -1.82^{*}(\pm0.77) + 2.25^{**}(\pm0.81) \times pH + 1.83^{**}(\pm0.32) \times OM$	<0.0001	0.28
	$^{b}NEA = 2.74^{**}(\pm0.64) - 0.22(\pm0.08) \times H_{AOA} - 0.44(\pm0.25) \times H_{AOB}$	<0.0001	0.12

Abreviations: [a], regression model using abiotic parameters; [b], regression model using biotic parameters; AOA; ammonia oxidizing archaeal; AOB, ammonia oxidizing bacteria; *H*, Shannon diversity index; *A*, gene abundance; NEA, potential nitrifying enzyme activity; NO_3 , nitrate in mg kg^{-1} dw^{-1}; NH_4, ammonium in mg kg^{-1}; pH, soil pH; *OM*, organic matter in %; *clay*, clay content in % and *humidity*, soil moisture in %. Models were restricted to a maximum of two parameters. *P < 0.10, **P < 0.05.

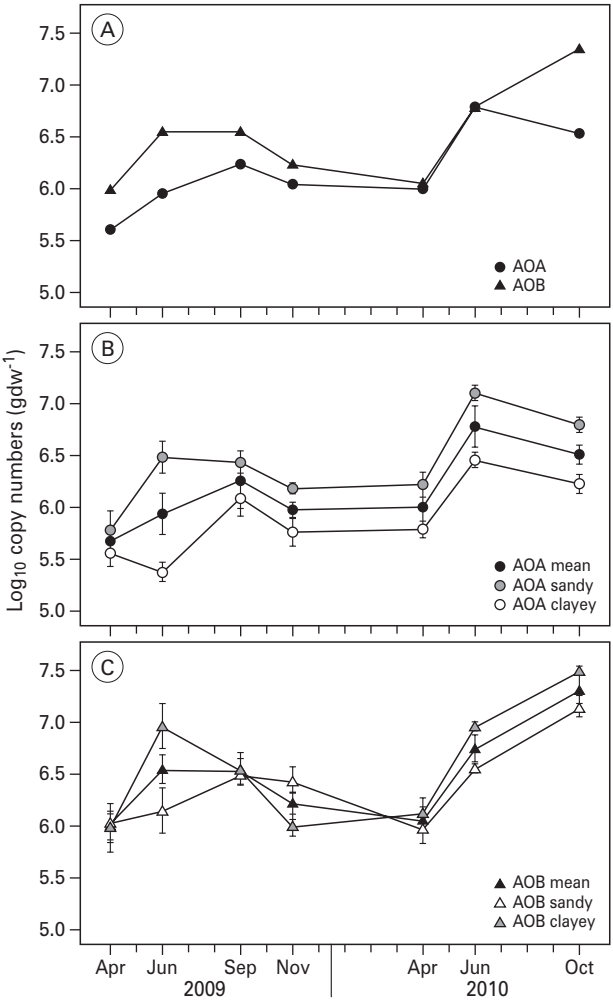

Figure 4.2. Amplitude of variation in the community size of AOA and AOB. Fluctuations in the community size were determined by real time quantification (qPCR) of *amo*A gene as a mean of all soils (A; $\Delta t_{AOA} = 1.21 \pm 0.08$; $\Delta t_{AOB} = 1.35 \pm 0.14$) and separated in sandy and clayey for both AOA (B; $\Delta t_{sandy} = 1.18 \pm 0.02$; $\Delta t_{clayey} = 1.25 \pm 0.02$) and AOB (C; $\Delta t_{sandy} = 1.45 \pm 0.30$; $\Delta t_{clayey} = 1.26 \pm 0.08$). Bars are standard errors.

for AOB. The dynamics of the AOA and AOB communities were addressed by moving window analysis (MWA), whose concept can be interpreted as the number of species that on average come to significant dominance at a given habitat, during a defined time interval. Our results showed that AOA and AOB had different patterns of variation. On average, the variability of AOB was

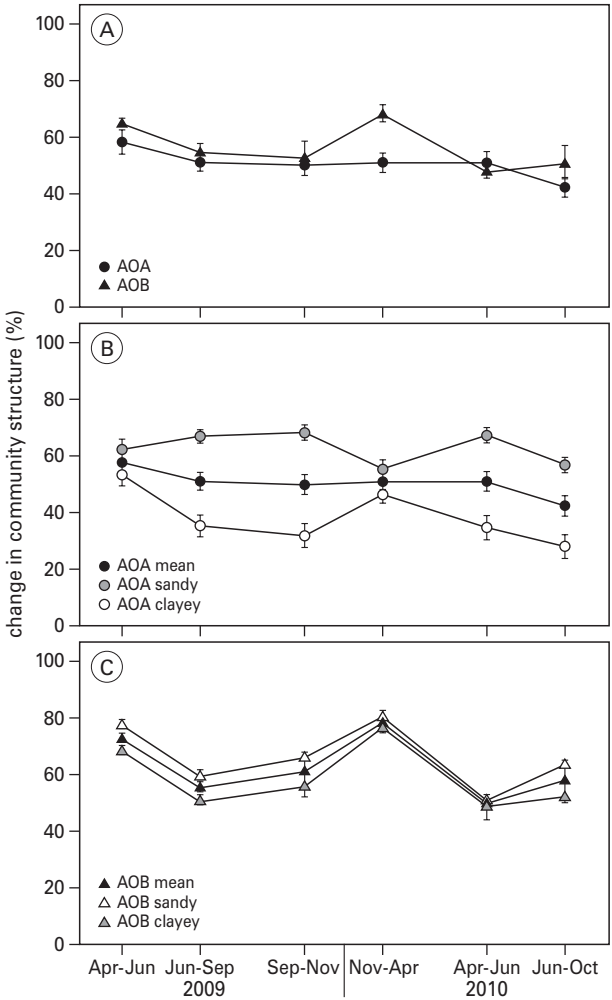

Figure 4.3. Amplitude of variation in the community structure of AOA and AOB. The fluctuations were determined by moving window analysis (MWA) (Marzorati *et al.*, 2008) based on DGGE profiles as a mean of all soils (A; Δt_{AOA} = 53.65 ± 3.37%; Δt_{AOB} = 62.76 ± 2.10%), and separated in sandy and clayey for both AOA (B; Δt_{sandy} = 60.27 ± 2.97%; Δt_{clayey} = 41.09 ± 3.92%) and AOB (C; Δt_{sandy} = 58.98 ± 3.01%; Δt_{clayey} = 66.55 ± 1.18%). Bars are standard errors. In the MWA each data point is in itself a comparison between two consecutive sampling times. Bars are standard errors.

higher than that of AOA populations (Δt_{AOB} = 62.76 ± 2.10% and Δt_{AOA} = 53.65 ± 3.37%; Figure 4.3A). Whereas for AOA higher variations were detected in the sandy soils (Δt_{sandy} = 60.27 ± 2.97%) compared to the clayey ones (Δt_{clayey} = 41.09 ± 3.92%; Figure 4.3B), for AOB communities the amplitude of variation

was higher in the clayey soils (Δt_{clayey} = 66.55 ± 1.18%) compared to the sandy ones (Δt_{sandy} = 58.98 ± 3.01%; Figure 4.3C).

Canonical correspondence analysis was used to investigate possible trends in the temporal changes in the community structures of AOA and AOB, and to test the significance of the influence of soil parameters on those changes. Although seasonality seemed to play a role in the distribution of both communities, no clear trend could be observed. In general, communities at the start of the growth season tended to cluster together (Figures S1 and S2). This was true especially for the AOB, in both sandy and clayey soils. The community structures of the AOA seemed to be more variable across the sampling times. Moreover, all soil variables measured apparently exerted significant effects on the AOA and AOB community structures. In order to determine the relative contribution of each soil parameter, we used variance partitioning to control for the effect of each individual parameter, when all others are defined as covariables in the constrained analyses (Leps and Smilauer, 2003). Considering the whole data set, soil parameters explained 33.4 and 49% of the variability in AOA and AOB community structures, respectively. In both cases, the percentage of clay, OM and soil pH were the most important parameters, explaining 18% (AOA) and 5.9% (AOB) of these variation.

Separating the soil in two groups, i.e. sandy and clayey ones, led to an increase in the overall percentage of variation explained by soil parameters for the AOA communities (76.6% and 79.4% for clayey and sandy soils, respectively). In clayey soils, the percentage of clay, OM and soil pH explained 45.5%, whereas for sandy soils, these three parameters explained 44.9% of the variation in community structure. Although the separation per soil type diminished the total percentage of variation explained by the soil parameters for AOB communities (15.8% and 17.1% for clayey and sand soil, respectively), it increased the percentage of variation explained by the percentage of clay, OM and soil pH to 10.7–10.8%.

To gain larger fine-scale taxonomic resolution of the archaeal and bacterial communities, we constructed sixteen clone libraries based on the *amo*A gene, from the eight soils. Phylogenetic analyses of the archaeal *amo*A fragments revealed that all clones were related to sequences of uncultured crenarcheaeota obtained in earlier environmental studies. All sequences were found to cluster in a few groups denoted soil/sediment, sediment/soil and, to a lesser percentage, marine lineages (Figure 4.4A), with some site-dependent variability. Sequences from sandy soil sites were dispersed among sequences from soil/sediment, sediment/soil and marine clusters, whereas sequences from the clayey sites were mainly related to the soil/sediment and marine clusters (Figure S3). The soil type effect detected by multivariate analyses on DGGE data was also observed for clone libraries, as determined by UniFrac analysis of the archaeal *amo*A sequences (Figure 4.5A).

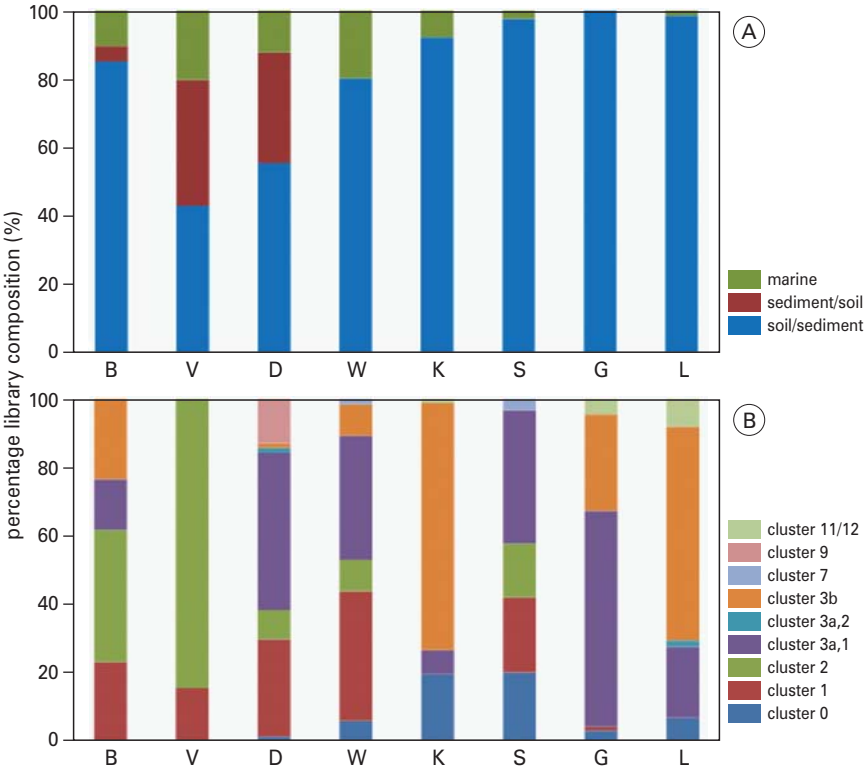

Figure 4.4. Diversity of archaeal (A) and bacterial (B) *amo*A sequences retrieved in this study from the eight soils. Archaeal and bacterial *amo*A clusters were classified according to Nicol e*t al.,* (2008) and Zhang *et al.,* (2009), respectively. See figure 4.1 for soil names.

Regarding the analysis of bacterial *amo*A gene fragments, almost all bacterial clone sequences represented *amo*A-like sequences that grouped with *Nitrosospira* clusters, one grouping with Nitrosomonas, although some of them showed no similarity with any known cluster (Figure S4). Most of the bacterial sequences were found spread over eight clusters and a high site-dependent variability was observed (Figure 4.4B). For instance, *Nitrosospira* cluster 3b was predominant in soils K and L, whereas cluster 11/12 comprised 40% and 90% of the sequences in soils B and V, respectively. We further found that the sequences tended to cluster according to soil type, being *Nitrosospira* clusters 1 and 2 mainly represented in the sandy soils and *Nitrosospira* cluster 0 mostly in the clayey soils. This was also confirmed by UniFrac analysis of the sequences (Figure 4.5B).

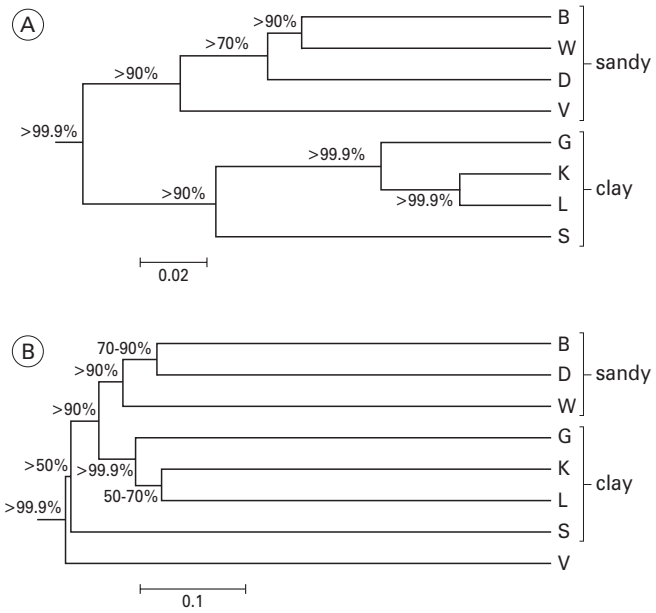

Figure 4.5. Dendrogram based on AOA (A) and AOB (B) *amo*A clone libraries, showing the differences in the community structure from eight soils. Distance matrices generated with UniFrac were used to cluster the soils using UPGMA; and jackknife analysis was used to evaluate how robust each environment cluster is to sample size and evenness. Numbers indicate the frequency with which nodes were supported by jackknife analysis.

Seasonal variation of NEA in relation to biological parameters

NEA was positively correlated with the abundance of AOA ($r = 0.74$, $P \leq 0.05$), but not with that of AOB (Table 4.4). Changes in nitrification rates were also significantly correlated with changes in the community structures of the AOA and AOB, which were observed to vary with season and soil type (Table 4.6 and Table S1). The analyses over time revealed high correlation values between NEA and AOA community structures at all times (except June 2010). These were season dependent, as correlations with the AOA communities were higher at the end of the season (September and November) and lower at the start (April and June). Correlations between activity and AOB community were higher at the start (June 2010) and lower at the end (September 2009 and October 2010)

Overall, NEA was affect mainly by the abundance and diversity (Shannon index from DGGE profiles) of AOA, which together explained 17% of the variation in nitrifying activities (Table 4.5). The results observed were similar for the sandy soils; however in the clayey soils only diversity, but not abundance, of AOB seemed to play a significant - although small - role (Table 4.5).

Table 4.6. Correlations between the community structure AOA and AOB, soil chemical parameters (pH, N-NH_4^+, N-NO_3^-, OM %, clay content % and water content %) and NEA (*Relate* Analysis) obtained with Primer-E (BEST Test), for all sampling times per soil and per soil type.

	NEA* ($\mu gN.h^{-1}\ gdw^{-1}$)	pH ($CaCl_2$)	N-NH_4^+ ($mg^{-1}\ kg$)	N-NO_3^- ($mg^{-1}\ kg$)	OM (%)	Humidity (%)
AOA structure across soils (*i*)						
Buinen	NS	NS	NS	0.49***	NS	NS
Valthermond	NS	0.48**	0.22**	0.12*	NS	0.19**
Droevendaal	0.19*	0.59***	0.16*	0.36***	0.24**	NS
Wildekamp	0.73***	0.58***	0.28**	0.44***	NS	NS
Kollumerwaard	NS	NS	0.15*	NS	0.33**	NS
Steenharst	NS	0.58***	NS	0.26**	0.22**	0.16*
Grebbedijk	0.56***	0.28**	0.15*	0.39***	0.15*	NS
Lelystad	NS	0.28**	NS	NS	NS	0.24**
Per soil type (*iii*)						
Sandy	0.26**	0.39***	0.53***	0.49***	0.43***	0.46***
Clayey	0.07*	NS	0.05*	NS	NS	NS
AOB structure across soils (*i*)						
Buinen	0.54***	0.12*	0.36***	0.31***	NS	NS
Valthermond	0.40***	0.37***	NS	NS	0.28***	0.25**
Droevendaal	0.42***	0.46***	0.22**	0.29**	0.29***	NS
Wildekamp	0.57***	0.24**	0.18*	0.19*	NS	NS
Kollumerwaard	NS	0.22*	0.27***	0.19*	0.40***	NS
Steenharst	0.58***	0.44***	NS	0.12*	0.32***	NS
Grebbedijk	NS	NS	0.32**	NS	0.16*	0.15*
Lelystad	NS	0.21**	0.25***	NS	NS	0.18*
Per soil type (*iii*)						
Sandy	0.26***	NS	0.09***	0.08***	NS	NS
Clayey	0.19***	NS	0.09***	NS	0.08*	NS
NEA across soils (*i*)						
Buinen	0.19*	0.16*	NS	NS	0.19	0.19*
Valthermond	NS	NS	0.26*	NS	0.24*	NS
Droevendaal	0.29**	NS	0.30**	NS	NS	0.29**
Wildekamp	0.19*	NS	NS	NS	NS	0.19*
Kollumerwaard	NS	0.21*	NS	NS	NS	NS
Steenharst	NS	NS	NS	0.37**	NS	NS
Grebbedjik	NS	NS	0.31**	NS	NS	NS
Lelystad	NS	NS	0.20*	NS	NS	NS
Per soil type (*iii*)						
Sandy	0.48**	S	NS	NS	NS	NS
Clayey	NS	0.05*	NS	NS	NS	NS

Abbreviations: NEA, Nitrifying enzyme activity; AOA, ammonia oxidizing archaeal; AOB, ammonia oxidizing bacteria;
NS, not significant ; *** $P < 0.001$, ** $P < 0.01$; * $0.01 < P < 0.05$.

Discussion

Temporal and spatial variation in potential nitrification rates in relation to chemical parameters

In general, the rates of potential nitrification in soil have been found to vary greatly, whether in an agricultural field, natural grassland or a forest soil, with observed amplitudes of variation being soil-dependent. Rates of 40 to 132 μg NO_2^- -N h^{-1} have been observed in acid agricultural soils in China (Yao *et al.* 2011) and of 20 to 120 μg NO_2^--N kg^{-1} h^{-1} in a forest soil in the UK (Wheatley *et al.*, 2003). In a field under intensive cultivation in a wheat-barley-potato rotation, rates were found to vary from around 5 to 127 μg NO_2^--N kg^{-1} soil h^{-1} (March to August 1998), and from 120 and 180 to 20 μg NO_2^--N kg^{-1} soil h^{-1} (June to January 2000). In the soils analyzed by us, the nitrification rates varied significantly over time, although the values were much lower than the above-mentioned ones, from 0.59 μgN.h-1 to 0.79 μgN.h^{-1}. This variation could be mainly attributed to two soil parameters, soil pH and soil texture. Interestingly, positive correlations between pH and NEA were consistently found, which might be explained by the fact that at lower pH values an increasingly higher number of ammonia oxidizers is inhibited (Webster *et al.*, 2002).

This indicates that patterns of NEA become even more complex when including the perspective of time. Nevertheless, such overall process parameters are important, as they constitute the "normal" amplitude of variation found across soil systems. Regarding the influence of soil parameters, several soil factors are known to influence the potential nitrification rates. For instance, the rates of potential nitrification were found to increase with decreasing salinity (Caffrey *et al.*, 2007) and with increasing temperature up to 30ºC (Tourna *et al.*, 2008). The rates are known to be significantly reduced in acid soils (de Boer and Kowalchuck, 2001), although only a slightly significant negative relationship between nitrification and pH was observed in organic soils (Booth *et al.* 2005).

Abundance and structure of ammonia oxidizing communities as affected by soil chemical parameters

It is important to understand how the nitrification process is impacted by soil conditions, and also how and to what extent the structure, composition and abundance of the ammonia oxidizing communities are affected, as the latter may coincide with altered rates. The population sizes of the AOA and AOB across soils and times were found to be within the range observed in other soil systems, i.e. XD to Y (Wessen *et al.*, 2011; Hallin *et al.*, 2009; Shen *et al.*, 2008). However, the AOA/AOB ratio's observed in our study were lower than those previously reported (Leininger *et al.*, 2006). In recent work, levels of 10^6 to 10^7 *amo*A gene copy numbers per g dry soil have been observed for the AOA and AOB in agricultural soils (Wessén *et al.*, 2011; Gubry-Rangin *et al.*, 2010),

although AOA numbers of up to 10^8 and AOB numbers of 10^7 have been observed in different agricultural soils (Leininger *et al.*, 2006). Moreover, *amo*A gene numbers as low as 10^4 have been found for AOB in flooded paddy soils (Chen *et al.*, 2010) and non-fertilized agricultural soil (Leininger *et al.*, 2006). We found significant seasonal variation in the abundances of AOA and AOB. Temporal variations in the abundances of AOB and AOB were also observed in a two year-study of the influence of different soil management techniques (Le Roux *et al.* 2008). Moreover, such variations were already hypothesized to occur in grassland and cropping systems (Berg and Rosswall, 1987).

Several soil factors, such as water content, seasonality and fertilizer type, are thought to affect the population sizes and community structures of ammonia oxidizers in soil (Nugroho *et al.*, 2006; Schmidt *et al.*, 2007; Hansel *et al.*, 2008). In this study, a highly positive correlation of soil pH with the abundance of AOA, but not with the abundance of AOB, was observed. Although soil pH is known to drive changes in the AOA and AOB communities (Nicol *et al.*, 2008, Erguder *et al.*, 2009), its effects are still controversial, as decreases in AOA abundances have been observed both with decreasing (Hallin *et al.*, 2009), and increasing soil pH (Nicol *et al.*, 2008). Other factors, such as soil moisture and nitrogen availability, are also known to influence the ammonia oxidizing communities (Hallin *et al.*, 2009; He *et al.*, 2007). It has previously been shown that AOA are more abundant in soils with lower levels of available nitrogen, whereas AOB become more abundant in soils under higher levels (Jia and Conrad, 2009). However, in the current study, none of these factors (moisture and N availability) determined the AOA/AOB community sizes. This may indicate either that the abundances are not affected by these drivers or, most probably, that the drivers are not the same across soils. Several previous studies proposed soil type to be the primary determinant of the bacterial composition in arable soils (Girvan *et al.*, 2003; Gelsomino *et al.*, 1999), but only few studies have addressed the effect of soil type on AOA and AOB abundance. Wessen *et al.* (2011) found that the abundance of AOA was negatively affected by clay content, which could be indicative of the AOA being less abundant in the supposedly nutrient-rich environments. However, in our study we did not find significant correlations between AOA abundance and clay content.

The community structure analyses by MWA indicated higher changes for the AOB, whereas the observed changes were lower for AOA. MWA describes the stability and species turnover over time; hence, a 65% change in AOB community structure between April and June means that from April to June the AOB community was very dynamic. In fact, the two communities shared only 35% of phylotypes and 65% changed over this time period. The AOA community was less dynamic than AOB over this time period, as shown by the lower percentage of change. Multivariate analysis revealed that the variables that contributed the most to changes in the system were soil clay content, OM and pH. In fact, taking

the differences in soil texture into account, we increased the resolving power of the method, allowing the detection of significant differences in the pattern.

An effect of soil texture on the phylogenetic make-up of the AOA was also observed, as sequences from the sandy soils formed a cluster that was separate from those from the clayey ones (supported by UniFrac). Analyses of the bacterial *amo*A genes showed a dominance of *Nitrosospira* clusters 3a.1 and 3b, which was mainly due to their dominance in the clayey soils. Conversely, in the sandy soils, *Nitrosospira* clusters 1 and 2 were dominant. This is consistent with findings by Stephen *et al.* (1996), who detected a dominance of *Nitrosospira* cluster 3 in pH-neutral agricultural soils versus *Nitrosospira* cluster 2 in more acidic soils; this followed a classification of AOB clusters defined in other studies (Avrahami and Conrad, 2003).

Exploring the effect of different aspects of ammonia-oxidizing communities on nitrification rates

Strong correlations were found between NEA and AOA abundances (Table 4.4). Moreover, when studying AOA and AOB community structures, higher correlations of NEA were found with AOA than with AOB at all times (Table S1). In contrast, Morimoto *et al.* (2011) found that nitrification rates in a low-humic Andosol soil correlated more with the abundance of AOB, suggesting that the relative importance of AOA or AOB to nitrification is site -dependent. The composition of the AOA and AOB communities, and their potential niches, also play roles in soil nitrification rates. Phylogenetic analyses of archaeal *amo*A genes showed that the sequences retrieved were quite similar to sequences found in previous studies (Figures S3 and S4). Moreover, the diversity was low, as indicated from rarefaction analysis. Although no study has been able to clearly link the rate of nitrification with the presence of distinct AOA or AOB groups, it was recently reported that higher nitrification rates were observed in sediments dominated by phylogenetically more diverse archaeal *amo*A sequences (Wankel *et al.*, 2010). This goes against our findings, which revealed that higher nitrification rates occurred in less diverse soil assemblages, indicating that just a few dominant types maybe be responsible for the nitrification process in these soils.

Establishing the NOR of nitrification in agricultural soils

In the past decades, a lot of attention has been given to the effect of external disturbances on soil microbial communities (Drenovsky *et al.*, 2010; Bardgett *et al.*, 2008; Wertz *et al.*, 2007; Mendum 2002). For instance, the effects of alien plant species or GM plants on the sizes, structures and compositions of microbial communities (Inceoglu *et al.*, 2011) have been addressed. This applies also to the introduction of new plant cultivars (GM or non GM), and any concomitant changes in agricultural practices, such as mechanization, different ploughing

regimen, planting times and pest controls, which might cause disturbances in soil microbial processes. An elegant approach to determine the effects of such factors on field soil is to evaluate whether their strength is enough to affect processes in such a way that these fall outside of what would be considered normal. In order to do so, we need to know the NOR of the process under "normal" or natural conditions. In the context of our work, we propose the definition of a NOR for agricultural soil by determining the highs and lows in selected processes relevant for ecosystem functioning. Thus, a range of soil attributes or properties (indicators) are selected, which are representative of a process that is sensitive to external drivers and easily measured and whose changes can be monitored through time, as previously suggested (Bruinsma *et al.* 2003, Kowalchuk *et al.* 2003). Second, it is crucial to take measurements from long-term datasets, possibly across several sites, to enable the capturing of environmental fluctuations that are independent of spatial and temporal scales. Third, only after implementing the appropriate NOR in a model can the concept of an overall soil NOR be fully operational.

We considered the oxidation of ammonia as such a sensitive process, and established the natural fluctuations by analyzing community structure, abundance and activity of ammonia oxidizers in eight soils over two year. We took into account seasonal influences, management practices, addition of fertilizers and crop rotation, representing the "normal" conditions. We observed that the drivers of the changes in structure, abundance and activity were mainly clay content and soil pH, although other soil parameters were also found to affect the structures of these communities, e.g. nitrate and ammonium. All analyses performed indicated a strong effect of soil type, roughly defined in sandy and clayey. Although this division is somewhat loose, and other factors such as pH co-vary with soil type, it enabled us to detect significant differences in the NOR. The relevance of soil type has also been found in studies focusing on macroorganisms, in which gene expression of the soil-dwelling collembolan *Folsomia candida* was differentially regulated in clayey versus sandy soil (de Boer *et al.*, 2011). These results suggest that differences in chemical composition observed between sandy and clay soils are of great relevance when studying soil organisms in general, suggesting that a soil-type-dependent NOR should be envisaged. One can argue that the timeframe used in our study is relatively short for definitive conclusions. However, the soils used in this study are subjected to the same agricultural practice for many years already, and thus a two-year study was considered a sufficiently sound first step allowing the definition of the variation that might be considered normal for these sites. Moreover, from the data we may already glean a basis for a possible NOR.

Overall, the NOR of potential nitrification was also different between sandy and clayey soils, being lower and less variable over time in the sandy than in the clayey soils. Moreover, the biological and chemical parameters measured

were better able to predict nitrification rates in sandy soils. Both ammonia-oxidizing communities were sensitive to the parameters associated with soil type, and fluctuated differently among each other, as well as within soils with contrasting texture and pH. MWA showed the AOB communities to fluctuate more, indicating a more dynamic community with higher species turnover than AOA. Furthermore, the diversity of both communities differed greatly between sandy and clayey soils. This soil-type-specific response indicated that different aspects of the bacterial and archaeal ammonia oxidizers should be taken into account when evaluating the effect of external disturbances on nitrification. Understanding the normal fluctuations of these soil communities and determining how environmental variations structure them will allow the provision of a key monitoring tool (Magurran *et al.*, 2010). In this context, the NOR of soil functioning will allow us to define normality and to grasp the mechanisms responsible for variation, enabling us to describe the impact of perturbations on the process measured.

Conclusion

To be able to assess the impact of disturbances on soil microbial community structure and function, it is imperative to obtain complete knowledge of the "normal" sources of variation, the extent to which they influence soil microorganisms and the possible outcomes of this interaction. Accordingly, the collection of a large data set, like the current one, should be encouraged for both natural ecosystems and agricultural areas. These types of data will be of key relevance when evaluating the impact of GM plants or global change on soil ecosystem services. We propose here that, to establish the NOR of nitrification in agricultural soils, both the AOA and AOB abundances and community structures should be considered in addition to the nitrifying activities. The conspicuous differences regarding soil type dictate the establishment of NORs per soil type. However, field studies comparable to the current one, performed across time, remain necessary to evaluate the extent and direction of the variations that underpin the NOR of nitrification.

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Table S1. Correlations between the similarity matrices of community structure AOA and AOB, soil chemical parameters (pH, N-NH_4^+, N-NO_3^-, OM %, clay content % and water content %) and NEA (Relate Analysis; NEA from September 2009 and October 2010) obtained with Primer-E (BEST Test), for all sampling times.

	NEA ($\mu gN.h^{-1}.gdw^{-1}$)	Soil type (ANOSIM)	pH ($CaCl_2$)	N-NH_4^+ (mg^{-1} kg)	N-NO_3^- (mg^{-1} kg)	OM (%)	Clay (%)	Humidity (%)
Community structure (a) across season (*ii*)								
AOA DGGE								
June 2009	NA	0.74***	0.18**	0.12*	0.31***	0.16*	0.67**	0.09*
Sept 2009	0.49***	0.63***	0.62**	0.36**	0.20**	0.19**	0.37**	0.15*
Nov 2009	0.39***	0.57***	0.54***	0.33***	0.12*	0.12*	0.35**	0.21**
April 2010	0.28***	0.97***	0.59***	0.13*	0.33***	0.23***	0.23***	0.33***
June 2010	0.25***	0.50***	0.46***	0.16**	0.38***	NS	0.18**	NS
October 2010	0.38***	0.79***	0.30**	0.23*	0.15**	0.17**	0.13*	0.22**
AOB DGGE								
June 2009	NA	0.16**	0.24***	0.33***	0.29***	0.23***	0.10**	NS
Sept 2009	0.19**	0.21***	0.29***	0.30***	NS	NS	0.25***	NS
Nov 2009	0.25***	0.10*	0.14**	0.09**	NS	NS	NS	NS
April 2010	0.23***	0.37***	0.31***	NS	NS	0.19*	0.25***	NS
June 2010	0.37***	0.54**	0.44***	0.23*	0.29***	0.25**	0.42***	NS
October 2010	0.17***	0.25**	0.41***	NS	0.15*	NS	NS	NS

Abreviations: ANOSIM, Analysis of Similarities, NEA, Ammonia oxidizing enzyme activity; AOA, ammonia oxidizing archaeal; AOB, ammonia oxidizing bacteria; DGGE, denaturing gradient gel electrophoresis; NA, not analyzed; NS, not significant ; *** $P < 0.001$, ** $P < 0.01$; * $0.01 < P < 0.05$. In April 2009 only four soils were measured and were therefore excluded from further analysis.
The relationship between soil physico-chemical parameters and the community structure of ammonia oxidizers were obtained Global Best test, and the effect of soil type by ANOSIM. Values are Global R values (sample statistic). Correlations between NEA and the community structure of ammonia oxidizers were obtained by RELATE Analysis, where values are Spearmans' Rho values (sample statistic).

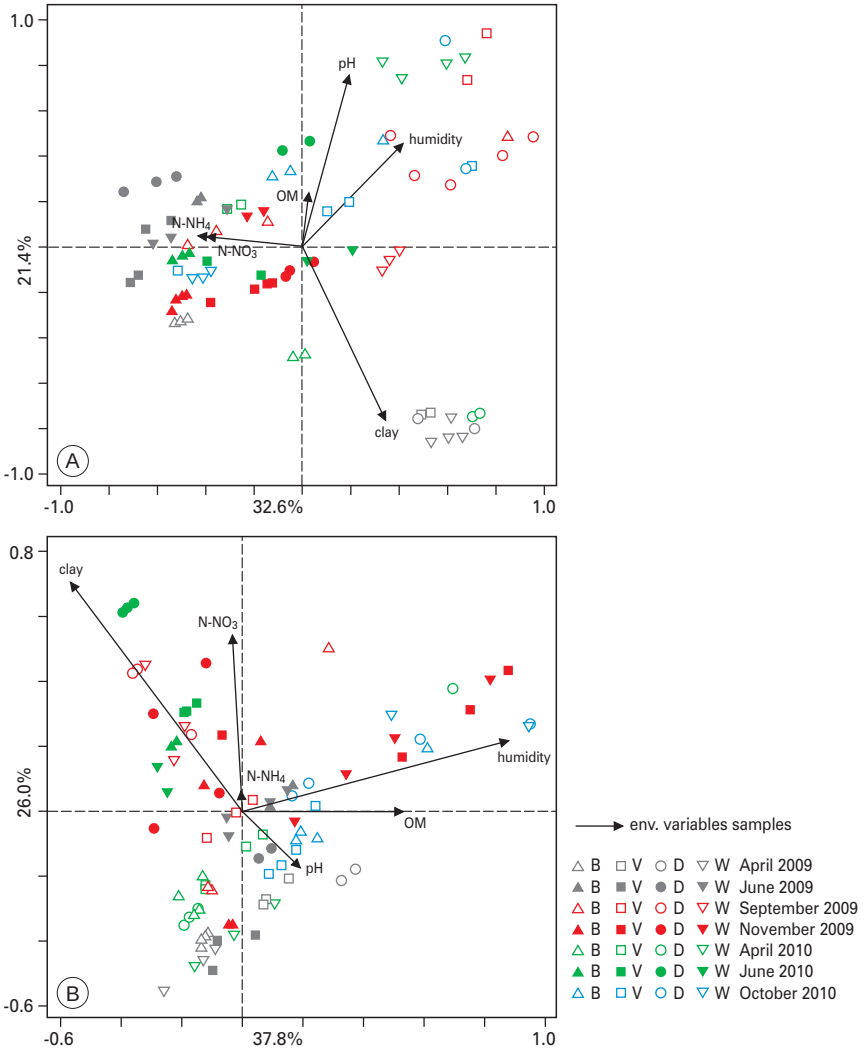

Figure S1. Biplots of canonical correspondence analysis of archaeal *amo*A gene (A) and beta-proteobacterial 16S rRNA gene (B) from DGGE data obtained from four sandy soils over two years. Physico-chemical data, soil moisture (Humidity), soil nitrate (N-NO3), soil ammonium (N-NH4), organic matter (OM), clay content (clay), soil pH (pH) are presented with black arrows.

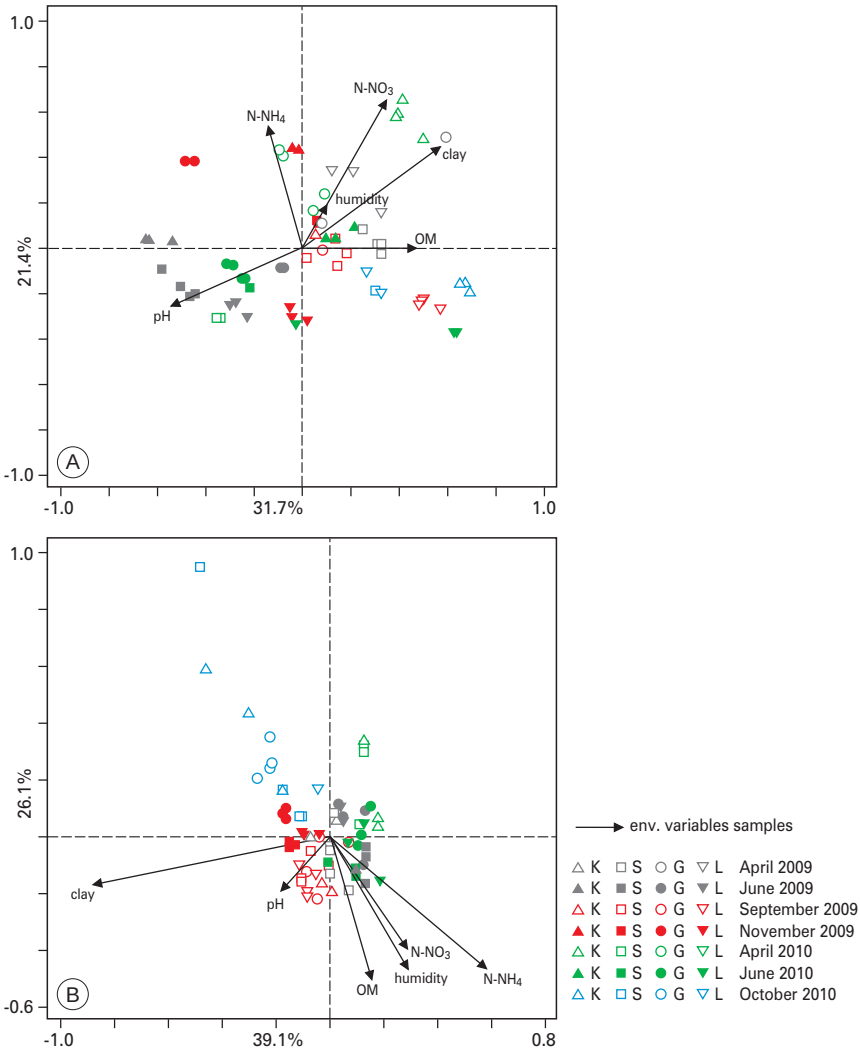

Figure S2. Biplots of canonical correspondence analysis of archaeal *amo*A gene (A) and beta-proteobacterial 16S rRNA gen (B) from DGGE data obtained from four clayey soils over two years. Physico-chemical data, soil moisture (Humidity), soil nitrate (N-NO3), soil ammonium (N-NH4), organic matter (OM), clay content (clay), soil pH (pH) are presented with black arrows.

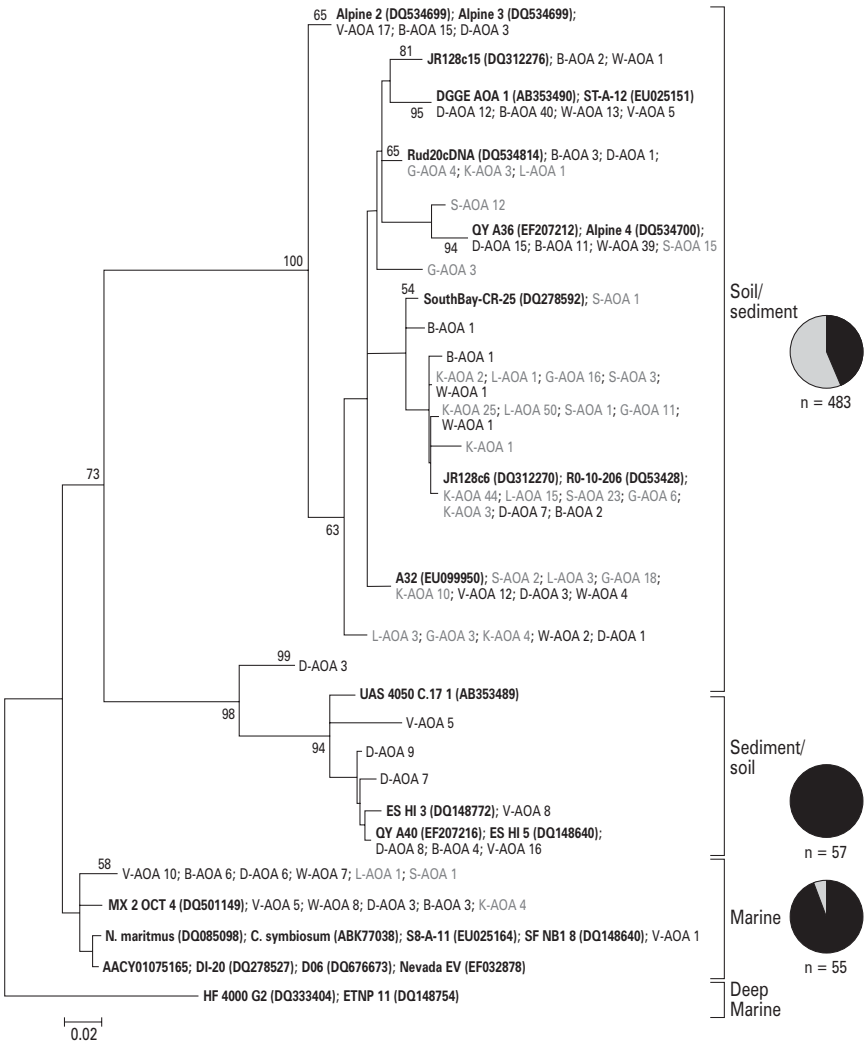

Figure S3. Phylogenetic analysis of 625 archaeal *amo*A partial amino acid sequences retrieved in this study from the eight soils. Sequences from sandy soils are represented in black and sequences from clayey soils in grey. Clades were classified according to Nicol *et al.,* (2008). Pie charts represent the percentage of sequences found in sandy and clayey soil and N values are total number of sequences found in the clade. Bootstraap support (>50) represent values from Neighbor-joining, using JTT substitution model (1000 replicates and 8 gamma rates; expressed as percentage). First letters represent the soil of origin followed by the number of clones found in that specific soil. Reference sequences are in bold described as 'Name (acession number)'. The tree was rooted with two sequences within the deep marine water clade.

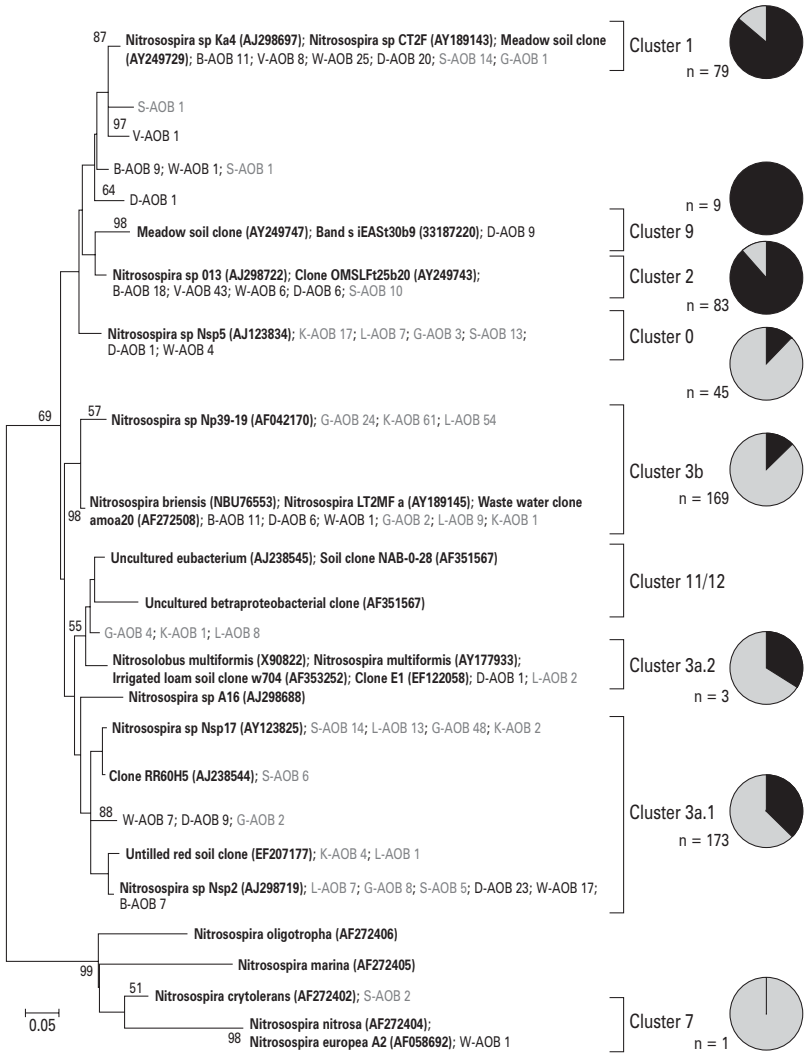

Figure S4. Phylogenetic analysis of 583 bacterial *amo*A partial amino acid sequences retrieved in this from the eight soils. Sequences from sandy soils are represented in black and sequences from clayey soils in grey. Clades were classified according to Zhang *et al*., (2009). Pie charts represent the percentage of sequences found in sandy and clayey soil and N values are total number of sequences found in the clade. Bootstraap support (>50) represent values from Neighbor-joining, using JTT substitution model (1000 replicates and 8 gamma rates; expressed as percentage). First letters represent the soil of origin followed by the number of clones found in that specific soil. Reference sequences are in bold described as 'Name (acession number)'. The tree was rooted with four sequences within the *Nitrosomonas* clade.

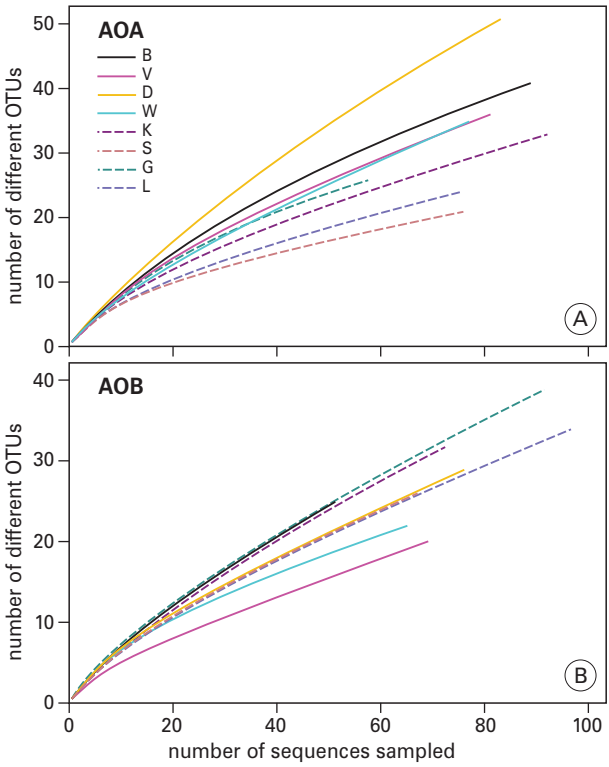

Figure S5. Rarefaction curves of observed operational taxonomic units (OTU) based on archaeal (A) and bacterial (B) *amo*A sequences retrieved from the eight soils, determined by DOTUR (Schloss and Handelsman, 2008). See legend from figure 4.1 for soil names.

Chapter 5

Temporal dynamics of abundance and composition of N-fixing communities across agricultural soils

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Unpublished manuscript

Abstract

In order to understand the functioning of diazotrophic communities and their resilience to external changes, we quantified the abundance of the *nif*H gene and characterized the community structure and composition based on it, across four representative Dutch soils during one growing season. Our results showed that, whereas higher copy numbers were observed in soils with higher pH than in those with lower pH, lower numbers occurred as related to increased nitrate and ammonium levels. Ordination of PCR-DGGE generated patterns and nifH gene pyrosequencing indicated that the N fixers are highly dynamic across time and soils. Per soil, very different communities were found at each sampling time. Deep sequencing of the *nif*H gene revealed that the functional diversity was high for most of the soils. Moreover, it increased from April on towards the end of the season. The dominant nifH carrying class was affiliated with *Alphaproteobacteria*, followed by *Beta*- and *Gammaproteobacteria*. The abundances of some nifH types were low throughout the season, such as found for *Acidithiobacillus* and *Anaeromyxobacter* nifH types, whereas others increased their abundances greatly over time (e.g. *Paenibacilus*, *Burkholderia*). From the environmental variables that were analysed, N availability (nitrate and ammonium) was identified as the main driver of variations in the N-fixing community structure and composition. This was followed by soil pH and clay content, which acted particularly on the evenness of the N fixing community.

Introduction

The biological nitrogen cycle is one of the most significant nutrient cycles in the terrestrial ecosystem. Among its processes, biological nitrogen fixation (BNF), the reduction of atmospheric N_2 gas to biologically available ammonium, is a key process, as it replenishes the pool of biologically available nitrogen that is lost to the atmosphere via anaerobic ammonium oxidation and denitrification (Capone and Knapp, 2007). BNF is performed by diazotrophs, a highly diverse group of microorganisms. It is widely distributed across bacterial and archaeal taxa (Dixon *et al.*, 2004), which harbor the *nif*H gene, one of the genes encoding the structural part of the enzyme nitrogenase. The *nif*H gene is a suitable marker to study the diversity and composition of N fixers without the need of cultivation (Hsu and Buckley, 2009).

Nitrogen cycling in traditional agricultural fields relies on biological N fixation, which occurs primarily (but not exclusively) by diazotrophic bacteria in symbiosis with legumes (Peoples *et al.*, 1995). It has been suggested that symbiotic N-fixers can fix up to 150 kg N/ha/year in some ecosystems (Reed *et al.*, 2011). By contrast, all estimates of the contribution of free-living nitrogen fixation are much lower (0 to 60 kg N/ha/year) (Burgmann *et al.*, 2004). However, these can also be significant and even critical under certain conditions, depending on the environment (Roper and Smith 1991; Cleveland *et al.*, 1999; Gupta *et al.*, 2006).

It is commonly known that the local environment in which soil organisms coexist often changes with time (Schloter, 2003), which influences how they structure themselves and also how they perform their functions. Furthermore, global change will severely affect N turnover in soils (Ollivier *et al.*, 2011), impacting all processes of the nitrogen cycle to an unknown extent. In this sense, several classes of nitrogen-fixing bacteria might be greatly affected, as these are known to be highly sensitive to perturbation (Pankhurst et al, 1995; Doran and Safley, 1997). Several environmental factors have been suggested to influence N fixation in soils, including soil moisture, oxygen, pH, carbon quantity and quality, nitrogen availability (Hsu and Buckley, 2009), soil texture and aggregate size (Poly *et al.*, 2001b) and clay content (Roper and Smith, 1991). Agricultural practices, such as fertilization and ploughing, also play a major role as determinants of bacterial community structure in soil (Patra *et al.*, 2006; Salles *et al.*, 2006; Wakelin *et al.*, 2009). Therefore, we hypothesize that the abundance and diversity of diazotrophs would be lower at the beginning of the season (April and June), when fertilizers are applied, compared to October.

The fluctuations in local conditions may cause disturbances that affect the soil microbial community structure and composition (Hooper and Vitousek, 1997; Tilman *et al.*, 1997) at different temporal and spatial scales. Several studies have assessed temporal variation in nitrogen fixation rates within ecosystems,

finding up to 50% variation among seasons (Reed *et al.*, 2007; Perez *et al.*, 2004; Steward *et al.*, 2011). These temporal variations are likely driven by variation in light, precipitation (Bentley *et al.*, 1987) and temperature (Mergel *et al.*, 2001). Changes in nutrient availability may also alter the rates of nitrogen fixation through changes in community composition (Hsu and Buckley 2009; Wakelin *et al.*, 2007; Lindsay *et al.*, 2010). For instance, the expression of the *nif*H gene was found to be downregulated under low P conditions (Orchard *et al.*, 2009). Moreover, Lindsay *et al.* (2010) observed a negative correlation between *nif*H gene abundance and high N concentrations in a managed ecosystem.

Understanding temporal and spatial patterns in the abundance and distribution of communities has been a fundamental quest in ecology. In order to understand the functioning of microbial communities and their resilience to external changes, a key issue is to assess the community composition, quantify individual microbial population sizes, and study fluctuations thereof (van Elsas *et al.*, 2000; Hartmann and Widmer, 2006). Furthermore, a thorough description of the natural variation of the nitrogen fixing community in a wide range of soils is still missing. In this study, we analyzed four different soils at three times over one growing season, in April, June and October of 2010. The goal was to characterize the spatio-temporal variability of *nif*H-containing microorganisms in agricultural soils, as measured by changes in DGGE fingerprints, real-time PCR and *nif*H-gene pyrosequencing, relating this variability to environmental conditions.

Material and Methods

Sampling sites

Four soils from different sites in the Netherlands were sampled three times in 2010, April (after seedling), June (before flowering) and October (senescence stage). The fields are used for potato cropping and are under agricultural rotation regime with non-leguminous crops. Information on land-use and location is available (Table 5.1). The soils were chosen to represent different soil types (clay vs. sand) and present different chemical properties (Table 5.1 and Table S1). Bulk soil samples (4 replicates per soil; 0.5kg per replicate) were collected in plastic bags and thoroughly homogenized before further processing in the lab. A 100-g subsample was used for measuring ammonia oxidizing enzyme activity, molecular biology and soil chemical properties.

Soil chemical analysis and activity measurements

The soil pH was measured after shaking a soil/water (1:2, w:v) suspension for 30 min (Hanna Instruments BV, IJsselstein, The Netherlands). Gravimetric soil moisture conents were determined comparison of fresh and dried (105ºC; 24h) weight of samples. Organic matter (OM) content is calculated as the difference

between the initial and final sample weights of dried soil measured after 4 hours at 550ºC. Nitrate ($N\text{-}NO_3^-$) and ammonium ($N\text{-}NH_4^+$) were determined in $CaCl_2$ extracts by a colorimetric method using the commercial kits Nanocolor Nitrat50 (detection limit, 0.3 mg N kg^{-1} dry weight, Macherey-Nagel, Germany) and Ammonium3 (detection limit, 0.04 mg N kg^{-1} dry weight; Macherey-Nagel, Germany) according to Töwe *et al.* (2010) .

DNA extraction

DNA was extracted from 0.5 g soil using the FastDNA SPIN Kit for Soil (MP Biomedicals) according to manufacturer's protocol. Extracted DNA was then precipitated and concentrated with cold ethanol to remove impurities. DNA concentrations were determined using the Quant-iT™ PicoGreen® dsDNA Reagent (Invitrogen, Darmstadt, Germany) according to the manufacturer's protocol using a spectrofluorometer (Spectramax Gemini, Molecular Devices GmbH, Germany). The quality of extracted DNA was estimated by running on agarose gel based on the degree of DNA shearing (average molecular size) as well as the amounts of coextracted compounds.

Standard PCR amplification for DGGE analysis

PCR reaction for DGGE analysis was performed targeting *nif*H of nitrogen-fixing community. PCR of *nif*H genes was conducted using a nested PCR according to Diallo *et al.* (2004). PCR reactions and cycling conditions are described in Table S2. DGGE profiles were generated with the Ingeny Phor-U system

Table 5.1. Sample locations, environmental and biological data.

Sampling Location	Buinen (B)	Droevendaal (D)	Kollummerwaard (K)	Grebbedijk (G)
Soil type	Sandy loam	Sandy loam	Clayey	Clayey
Land use	Agricultural	Agricultural	Agricultural	Agricultural
Sampling coordinates	52°55′386″N 006°49′217″S	51º59′551″N 005º39′608″S	53°19′507″N 006°16′351″S	51º57′349″N 005º38′086″S
pH	4.40	4.93	7.40	7.20
Nitrate ($N\text{-}NO_3^-$) mg/kg	47.17	60.53	24.60	29.93
Ammonium ($N\text{-}NH_4^+$) mg/kg	9.23	12.30	8.40	15.13
OM (%)	4.00	2.83	4.20	5.40
Water content (%)	11.20	11.63	19.30	19.60
*nif*H-gene Abund. ($x\ 10^5\ gdw^{-1}$)	0.72	1.15	17.0	3.10

Values of environmental and biological data are average of each soil across the three sampling times.

(Ingeny International, Goes, The Netherlands). The PCR product (300 ng/lane) were loaded onto 6% (w/v) polyacrylamide gels, with a 40-65% denaturant gradient (100% denaturant corresponded to 7 M urea and 40% (v/v) deionized formamide). Electrophoresis was performed at a constant voltage of 100 V for 16 h at 60ºC. The gels were stained for 60 min in 0,5x TAE buffer with SYBR Gold (final concentration 0,5 µg/liter; Invitrogen, Breda, The Netherlands). Images of the gels were obtained with Imagemaster VDS (Amersham Biosciences, Buckinghamshire, United Kingdom).

Real-time quantitative PCR

The abundances of *nif*H genes in the soils samples were quantified using real-time PCR. For the *nif*H gene, the primers FPGH19 (Simonet *et al.*, 1991) and PolR (Poly *et al.*, 2001a) were used according to Pereira e Silva *et al.*, 2011. Cycling programs and primer sequences are found in Table S2. Standard curves were generated from serial dilutions of plasmid containing cloned *nif*H gene from *Bradyrhizobium liaoginense*, from 10^6 to 10^2 gene copy numbers/µl. Absolute quantification was carried out twice from each of the four soil replicates on the ABI Prism 7300 Cycler (Applied Biosystems, Germany). The specificity of the amplification products was confirmed by melting-curve analysis, and the expected sizes of the amplified fragments were checked in a 1.5% agarose gel stained with ethidium bromide. Possible inhibitory effects of co-extracted humid compounds were checked by spiking standard concentrations with serial dilutions of soil samples. No severe inhibition was observed at the working dilutions. The qPCR efficiency (E) was calculated according to the equation $E = 10^{[-1/slope]}$ (Ritz and Spiess, 2008) and was 1.92. The R^2 of all these standards was higher than 0.99.

Deep-sequencing and bioinformatic analysis of *nif*H gene

Diversity of nitrogen-fixing bacteria in the four agricultural soils was investigated by barcoded pyrosequencing approach. The total community DNA was amplified with *nif*H gene-specific primers PolF/PolR (Poly *et al.*, 2001) and RoeschF/ RoeschR (Roesch *et al.*, 2006) in a nested approach using the FastStart High Fidelity PCR system and PCR Nucleotide Mix (Roche Diagnostics GmbH, Mannheim, Germany). PCR conditions and primer sequences are found in Table S2. Triplicate PCR amplifications were performed on each soil DNA template and pooled. Primer dimers were removed by electrophoresis of PCR products on agarose gel, excision, and purification using Qiaquick PCR purification Kit (Qiagen). For 454 pyrosequencing of samples, adapters and sample-specific tags were added using custom primers in an additional PCR amplification of 20 cycles using the same PCR conditions. Amplicons were further purified with AMpure beads (Beckman Coulter) and pooled in an equimolar ratio as specified by Roche. Sequencing from 5′ (forward) and 3′ (reverse) ends of amplicons

was performed. Emulsion PCR, emulsion breaking of DNA-enriched beads, and sequencing runs of the amplicon pools were performed on a second-generation pyrosequencer (454 GS FLX Titanium; Roche) using titanium reagents and titanium procedures as recommended by the manufacturer. The 454-pyrosequencing data have been deposited in the National Center for Biotechnology Information (NCBI) under accession number XXXXXXX. Quality filtering of the pyrosequencing reads was performed using the automatic amplicon pipeline of the GS Run Processor (Roche) to remove failed and low-quality reads from raw data. Amplicon libraries of the nitrogenase gene (nifH) were explored using the FunGene Pipeline of RDP server (http://fungene.cme.msu/edu/FunGene Pipeline) using the default settings. Primer sequences were trimmed and reads of low quality and shorter than 350 bp were removed. Filtered nucleotide sequences were translated into amino acid and clipped at 108 bp. All subsequent analyses were done on amino acid sequences. By targeting a protein-coding gene, frame-shifts errors caused by insertions or deletions of bases, can be identified (Huse *et al.*, 2007). Sequences were then visually inspected and sequences having in-frame stop codon(s) were removed. The amino acid sequences were aligned by MUSCLE 3.8 (Gouy *et al.*, 2010).

Operational taxonomic units (OTUs) were then classified and rarefaction curves were constructed with DOTUR (Schloss and Handelsman 2005) using 90% amino acid sequence similarity cutoff (Palmer *et al.*, 2009; Mao *et al.*, 2011). Richness estimates and diversity indices were calculated for the total number of sequences as well as for the subsets normalized to the same number of sequences by the Perl script daisychopper.pl (available at http://www.genomics.ceh.ac.uk/ GeneSwytch/Tools.html; Gilbert *et al.*, 2009). Phylogenetic analysis was performed for clustered sequences at 90% similarity using CD-HIT (Li and Godzik, 2006), and with more than 10 sequences. The representative sequences were used to build neighbor-joining trees in MEGA5 (Tamura *et al.*, 2011). Normalized weighted UniFrac significance (Lozupone *et al.*, 2006) was calculated to evaluate differences between the *nif*H-gene communities and for clustering analyses based on the phylogenetic trees obtained in Mega5. The *nif*H representative sequences were blasted against a non-redundant protein sequence database using BLASTP.

Biotic-environment relationship

To test the influence of soil physicochemical parameters (environmental factors) on community data, forward selection was used on canonical correspondence analysis (CCA) or redundant correspondence analysis (RDA), depending on the gradient length observed on DCA. If it was longer than 3.5 a unimodal method (CCA) was used. Otherwise, with a gradient shorter than 3.5, RDA was chosen. The forward selection was used to select a combination of environmental variables that explained most of the variation observed in *nif*H-gene species

matrix. For that, all parameters except pH were log10-transformed, and the community matrices (obtained from DGGE bands as well as from pyrosequencing) were square-root transformed. Afterwards, a series of constrained CCA permutations was performed in Canoco (version 4.0 for Windows, PRI Wageningen, The Netherlands,) using automatic forward selection and Monte Carlo permutations tests (permutations = 999).

Statistical analysis

Computer-assisted analysis of DGGE profiles was performed using the GelCompar software (Applied Maths, Sint-Martens Latem, Belgium), to follow the structural changes on these communities over time. Similarity matrices were constructed based on Pearson's correlation coefficient and cluster analyses were done by unweighted pair group method with average linkages (UPGMA). The differences in soil chemical parameters and *nif*H gene abundances in the different soils over time were estimated with independent t-Tests.

Results

Seasonal variations of soil chemical properties

Soil pH, nitrate, ammonium and organic matter levels were determined in triplicate across all soil samples. Considering all soils, pH and OM levels were significantly higher ($P < 0.05$) in soils K and G than in soils B and D (Table S1). On the contrary, the levels of nitrate were higher in soils B and D (47.17 ± 0.37 and 60.53 ±1.66, respectively) compared to soils K and G (29.93 ± 1.15 and 24.60 ± 1.72, respectively). The first group (B and D) showed significantly higher values in June, whereas the latter (G and K) showed lower nitrate levels in the same period. The levels of ammonium also varied over the whole period. Relatively low values of ammonium were observed for all soils in October (on average 5.47 mg/kg ± 0.66) whereas higher levels were detected in April and June (on average, 14.63 mg/kg ± 2.66 and 14.01 mg/kg ± 0.61, respectively). Individual values for each soil at each sampling time can be found in Table S1.

Quantification of *nif*H gene copy numbers over time as related to soil variables

Overall, the *nif*H gene copy numbers fluctuated from 5.30×10^3 to 4.34×10^6 gdw^{-1} over the growing season, being significantly higher ($P < 0.05$) in October (1.38×10^6 gene copy numbers gdw^{-1}) compared to April (1.69×10^5 gene copy numbers gdw^{-1}) and June (9.13×10^4 gene copy numbers gdw^{-1}) (Fig 5.1). Analysis per soil revealed that the clayey soils K and G had significantly higher *nif*H gene abundance at all sampling times (1.0×10^6 gene copy numbers gdw^{-1} on average) compared to the sandy soils B and D (9.33×10^4 gene copy numbers

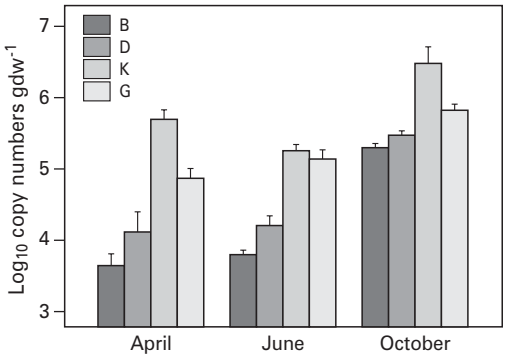

Figure 5.1. Changes in abundance of *nif*H gene in soils B, D, K and G collected in April, June and October of 2010. The copy numbers per gram of dry soil was estimated by real-time PCR. Soils: B, Buinen; D, Droevendaal; K, Kollumerwaard and G, Grebbedijk.

gdw^{-1} on average). Pearson's product-moment correlations were calculated to test the influence of soil variables on *nif*H gene abundance. Nitrate (–0.543, P = 0.000), ammonium (–0.565, P = 0.000) and pH (0.645, P = 0.000) were identified as main drivers of variation in *nif*H gene abundance.

Changes in the structure of the *nif*H gene carrying community in relation to soil variables

In order to characterize the dynamics of the N-fixing communities through time, we performed PCR-DGGE analyses based on the *nif*H gene. Analysis of the DGGE gels revealed that the numbers of bands per sample over time were in the range of 15±2, 12±1, 11±2 and 11±1, for soils B, D, K and G respectively. Moreover, these tended to decrease from April to October for the sandy soils, being constant in the clay soils. Analysis of the Shannon diversity index values for the whole dataset (data not shown) indicated that sample date had no significant effect on *nif*H diversity. However, the fingerprints of the *nif*H gene clustered into five groups at 38% similarity, which reflected an increasing effect of sampling time on the community structure (Fig. S1). This was confirmed statistically by forward selection in canonical correspondence analysis (CCA) (Fig. 5.2 and Fig. S2), indicating nitrate (28.6%, P = 0.04), ammonium (16.1%, P = 0.003), and clay content (24.4%, P = 0.048) as the main factors causing these shifts. Over time, an effect of soil type was also observed, especially in June and October. This occurred to a higher extent in the clayey soils (K and G), compared to the sandy ones (B and D).

Pyrosequencing analysis of the *nif*H gene diversity

To further understand the changes in the composition of the communities of

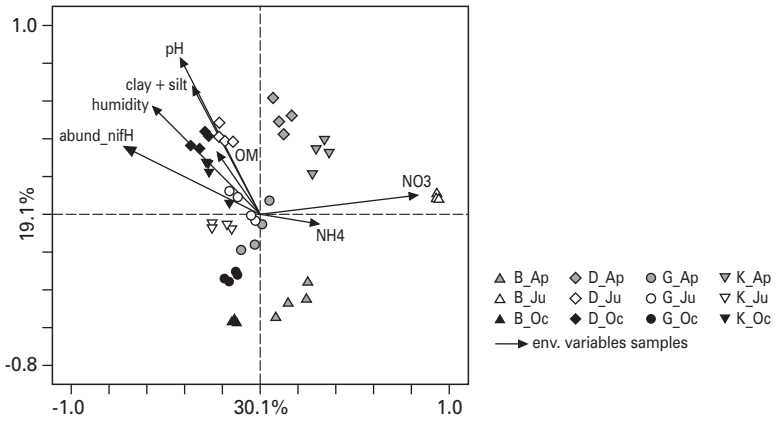

Figure 5.2. Changes in the structure of *nif*H-gene across different soils and sampling times, and the influence of environmental parameters, as revealed by Canonical correspondence analysis (CCA). The number in each axis shows the percentage of total variation explained. The length of the corresponding arrows indicated the relative importance of the geochemical factor in explaining the variation in microbial profiles. Soil samples were analyzed in four replicates at each sampling time. B, Buinen; D, Droevendaal; K, Kollumerwaard and G, Grebbedijk; Ap, April; Ju, June; Oc, October.

Table 5.2. Richness estimates and diversity indices for forward amplicon libraries at 90% similarity cutoff after random resampling of sequences to the same depth (1921 sequences).

Library Forward	OTUs[a]	Estimated OTU richness		Shannon[b]
		Chao1	ACE	
B_Ap	40	48.25 (42.16; 71.48)	53.53 (44.54; 80.31)	2.09 (2.03; 2.15)
D_Ap	61	95 (71.10; 175.41)	130.69 (107.37; 108.69)	2.79 (2.73; 2.85)
K_Ap	58	73.11 (62.76; 105.96)	123.4 (99.41; 177.03)	2.26 (2.19; 2.33)
G_Ap	58	75 (63.49; 110.55)	99.46 (82.48; 141.56)	2.60 (2.54; 2.66)
B_Ju	44	71.2 (52.63; 133.53)	59.55 (49.57; 87.45)	1.97 (1.91; 2.03)
D_Ju	52	71.43 (58.05; 114.31)	89.17 (74.59; 125.92)	2.56 (2.51; 2.61)
K_Ju	61	105.5 (75.89; 212.48)	256.19 (192.21; 375.32)	2.62 (2.55; 2.68)
G_Ju	58	85.14 (67.04; 139.51)	174.71 (142.76; 223.78)	2.54 (2.48; 2.60)
B_Oc	46	80.2 (56.82; 154.09)	134.79 (95.36; 222.26)	2.31 (2.25; 2.36)
D_Oc	79	122.5 (96.20; 188.97)	193.92 (160.06; 256.39)	2.78 (2.72; 2.85)
K_Oc	68	138.2 (92.71; 267.43)	173 (141.24; 237.01)	2.73 (2.66; 2.79)
G_Oc	49	70 (55.06; 121.68)	106.57 (88.35; 147.91)	2.53 (2.48; 2.58)

[a]Calculated with DOTUR at the 10% distance level.
[b]Shannon diversity index calculated using DOTUR (10%).
Values in brackets are 95% confidence intervals as calculated by DOTUR.

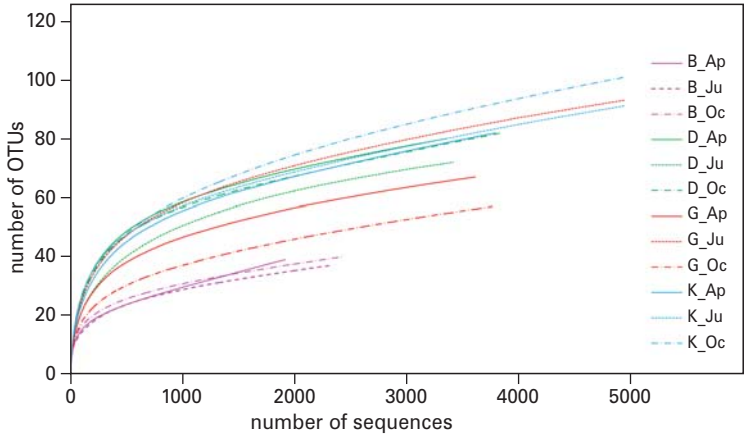

Figure 5.3. Rarefaction analysis of the diversities of *nif*H gene in the four soils and three sampling times. The OTUs were classified at 90% similarity cutoff based on amino acid sequences. B, Buinen; D, Droevendaal; K, Kollumerwaard and G, Grebbedijk; Ap, April; Ju, June; Oc, October.

N-cycling microorganisms in the four agricultural soils, the *nif*H gene was deeply sequenced using the pyrosequencing approach, in which forward and reverse reads were analyzed separately. In total, 91015 reads were obtained. The total numbers per sample are described in Table S3. These (transformed into amino acid sequences) ranged from 1,921 to 5,892 [average length 108 amino acids], corresponding to 40 – 120 OTUs (defined at a 10% sequence difference). Forward reads of the *nif*H amplicons yielded slightly more OTUs than did reverse reads, although the number of sequences was lower, indicating that the efficacy of forward reads of *nif*H is higher for diversity analysis than reverse reads. This was reflected in the reproducibility of the pyrosequencing runs, which resulted in a linear coefficient of $R^2 = 0.785$. Results obtained from the reverse reads showed similar trends to those obtained from forward ones. Therefore, we present the results referring to forward reads only.

In any community, estimates of species richness are always dependent on the sampling effort (Hughes *et al.*, 2001). Therefore, to allow for comparisons of diversity and richness among samples, the dataset was randomly resampled to the same sequencing depth (1,921 sequences per treatment), yielding adjusted total numbers of OTUs between 40 and 79 (Table 5.2). In addition, we found that the OTU numbers tended to increase with sampling time in three (B, D and K) out of four soils (Table 5.2). Rarefaction analysis of the libraries resulted in different saturation profiles, suggesting that the *nif*H diversity was higher in some of the soils (Fig. 5.3 and Fig. S3). The diversity of *nif*H gene sequences as described by the Shannon index was slightly higher towards the end of the sea-

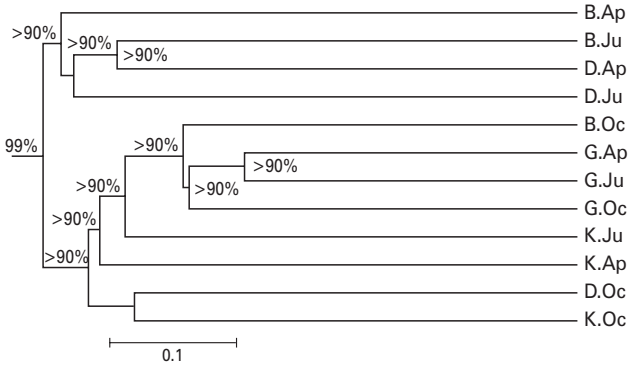

Figure 5.4. Dendogram from UniFrac analysis of *nif*H-gene libraries. Numbers indicate the frequency with which nodes were supported by jackknife analysis. Analyses were performed with respect to the abundance of each OTU (weighted data).

son. It was also raised in the clayey soils as compared to the sandy ones. These effects of sampling time and also soil type were confirmed by weighted Unifrac analysis and clustering of the sequences (which consider the evenness of the community) (Fig. 5.4).

Compositional diversity was assessed applying a 90% similarity cut-off. The lowest *nif*H gene diversity was observed in soil B, which was characterized mostly by *Bradyrhizobium*, *Burkholderia* and *Termochromatium* species. The highest diversity was observed in soil K, in particular in October sample, which was dominated by *Bradyrhizobium*, *Azomonas*, *Pelobacter* and *Methylomonas* species. However, after normalization of the data, the soil D in October appeared as the most diverse; it contained the *nif*H gene attributable to several species, e.g. *Herbaspirillum*, *Azospirillum*, *Bradyrhizobium* and *Methylomonas* species. An analysis at higher taxonomic rank per soil revealed that soils B and D were dominated by *nif*H containing putative members of the class *Alphaproteobacteria* (around 56%), whereas soils K and G were dominated by those from the Beta- (around 40%) and *Gammaproteobacteria* (around 28%).

Phylogenetic analyses based on amino acid sequences revealed that, in total, the retrieved *nif*H sequences varied substantially among soils and also over time (Fig. 5.5 and Fig. 5.6), being distributed among Alpha-, Beta-, Gamma- proteobacteria and Firmutes (Fig. 5.5 and Fig. 5.6). The most abundant class was Gammaproteobacteria, which were more than 95% similar to *Methylomonas*, *Methylobacter* and *Celerinatantimonas* species. Other groups were dominated by *nif*H genes typical for Alphaproteobacteria, specifically *Bradyrhizobium*, *Rhizobium* and *Azospirillum* species. The *nif*H genes affiliated to Betaproteobacteria were dominated by those of the order Burkholderiales. This included *Burkholderia*, *Azoarcus* and *Zoogloea* species.

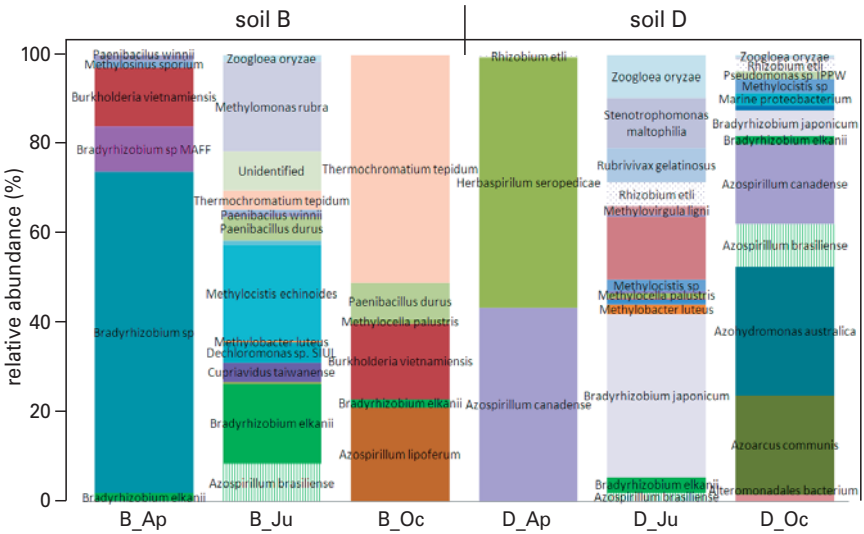

Figure 5.5. Taxonomic classification of the most abundant *nif*H-gene sequences associated with the two sandy agricultural soils and at three sampling times, April, June and October. Multi-colored charts at the legend are shown for each sample correspondingly.

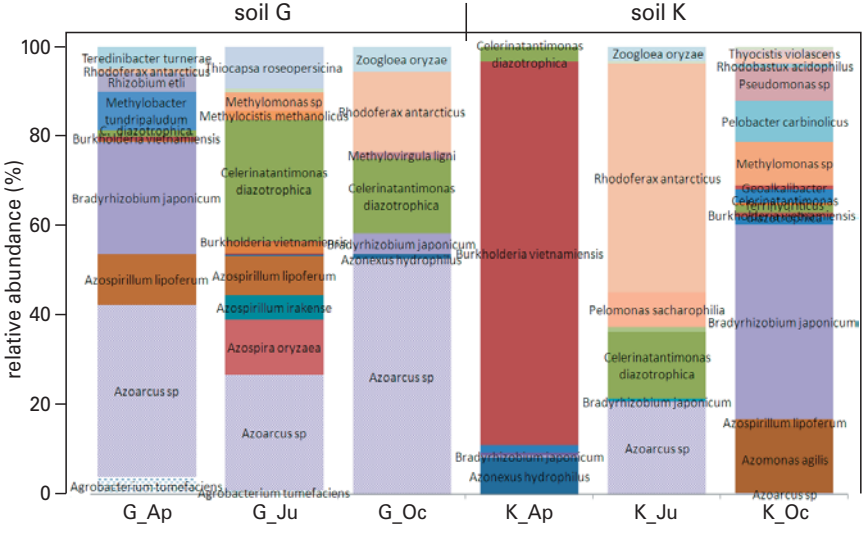

Figure 5.6. Taxonomic classification of the most abundant *nif*H-gene sequences associated with the two clayey agricultural soils and at three sampling times, April, June and October. Multi-colored charts at the legend are shown for each sample correspondingly.

Dynamics of abundant and rare *nif*H gene sequences

To understand which *nif*H gene types were affected by seasonality, we identified the OTUs that revealed abundance changes throughout the year. A very clear seasonal effect was observed in all soils. Interestingly, in particular cases a completely different community was present at each sampling time (Fig. 5.5 and Fig. 5.6). When time was considered alone, only 15 to 37% of the OTUs were found to be shared between sampling times. This percentage was much higher for soils G (37.25%) and K (36.60%), as compared with soils B (15%) and D (27.5%). These rather stable OTUs consisted of *nif*H genes typical for *Bradyrhizobium*, *Rhizobium*, *Azoarcus* and *Celerinatantimonas* species. Moreover, only a low percentage of OTUs was shared between soils at each sampling time, i.e. around 14-27%. In the April samples, the soils were dominated by just one or few *nif*H gene types. However, the *nif*H gene based diversity levels increased markedly towards October. This was very clear for soils B, D and K. A broader phylogenetic analysis revealed that samples at all sampling times were dominated by members of the *Alpha* subdivision of the *Proteobacteria*. In April, this represented around 50% of the N-fixing community across the board, followed by *Betaproteobacteria* (around 42%). In June, a similar high percentage of the alpha subdivion was found, but an increase from 7% to 31% was observed for *Gammaproteobacteria*. The latter group was still high in October (around 25%).

Some *nif*H types were commonly found to be spread over all the soils, e.g. *Bradyrhizobium*, *Azospirillum* and *Methylocystis*. However, some particular types could be identified as being typical for soil type and sampling time. For instance, *Paenibacillus durus*, *Termochromatium tepidum* and *Stenotrophomonas maltophilia* were observed only in soils B and D (sandy) (Fig. 5.4), whereas *Azoarcus* sp., *Burkholderia vietnamiensis* and *Celerinatantimonas diazotrophica* were mostly detected in soils K and G (clayey) (Fig. 5.5). Forward selection on RDA analysis identified ammonium (5%; P = 0.032), soil pH (4.2%, P = 0.042) and clay content (4.3%; P = 0.042) as the main drivers of the *nif*H gene compositional variation.

The satellite (rare) species, in contrast to the core (abundant) ones, are defined here as the OTU90% (Cluster at 90% similarity cutoff) that were represented by less than 10 sequences in the whole dataset. From the total number of cluster (441), 66% were represented by satellite species (288 clusters). We found that a large fraction of the bacterial nitrogen-fixing communities consisted of rare types, as was also observed for total bacteria communities (Galand *et al.*, 2009; Kirchman *et al.*, 2009; Inceoglu et al 2010). These rare members of the community also changed dramatically over time. Intriguingly, most of the rare types were affiliated with *Alphaproteobacteria*. Moreover, some remained rare (e.g. *Acidithiobacillus*, *Agrobacterium*, *Anaeromyxobacter*, *Azonexus*, *Pseudomonas* and *Skermanella*) whereas others became dominant at some point in time, e.g. some species of *Paenibacilus*, *Bradyrhizobium*, *Burkholderia* and *Stenotrophomonas*

(Fig. S4 and Fig. S5). Forward selection on RDA analysis identified the same environmental parameters as main determinants of the rare species, ammonium (5%; P = 0.017), pH (5.1%; P = 0.009) and clay content (5.8%; P = 0.005), with the addition of nitrate (5.3%; P = 0.018).

Discussion

Seasonal variations in *nif*H gene abundance

The population size of the N-fixing bacterial communities across soils and sampling times was found to be within the range observed in other soil systems (Hai *et al.*, 2009; Orr *et al.*, 2010; Huang *et al.*, 2011; Mao *et al.*, 2012). Significant seasonal fluctuations were observed in the abundance of the nitrogen fixers, with a tendency to increase towards October. Temperature is known to be one of the most important parameters influencing the soil bacterial community (Pettersson and Baath, 2003). As nitrogen fixation is an enzymatic process, it increases with increasing temperature, supporting the trend of a higher abundance from April (average air temperature 8ºC) to June (14ºC) and October (11º). Soil nutrient status (mineral N) also changes seasonally, with usually higher levels in June, after application of fertilizers but before the intake of available nitrogen by the root system of prevailing plants (Orr *et al.*, 2010). This variation in nitrogenous nutrients (ammonium and nitrate) strongly decreased *nif*H abundance, as previously reported in a managed ecosystem (Lindsay *et al.*, 2010), and as suggested by field evidence. The latter showed that free-living nitrogen-fixers in the soil will preferentially use fixed available N instead of fixing it (Barron *et al.*, 2008; Cusack *et al.*, 2009) due to the high energy demand of the latter process. However, the effect and availability are N is still controversial, as some studies have even reported higher *nif*H abundance in soils with high values of nitrate (Bothe *et al.*, 2002; Mergel *et al.*, 2001).

Soil pH was also identified as an important parameter, although its influence might be inherent in a soil type effect, as clayey soils have higher pH. Indeed, it has been shown that 70% of the free-living nitrogen-fixing bacteria are commonly found in soil clay fractions (Chotte *et al.*, 2002). Moreover, an effect of soil pH on the abundance of diazotrophs was previously observed (Pereira e Silva *et al.*, 2011); it co-varied with season, with stronger correlations at the beginning than at the end of the season.

Variations in *nif*H gene based PCR-DGGE patterns

Seasonal shifts were observed not only in the abundance of nitrogen fixers, but also in the community structures, as previously reported for those sites (Pereira e Silva *et al.*, 2011). In fact, it was found that around 60% of the diazotrophic community shifted from April to November. This structural change was found

to be not stochastic. A multivariate variation partitioning approach showed that variations in nitrate, ammonium and clay content were directly related to the changes (dominant OTUs). The effects of N availability on the diazotrophic community structures are as-yet not clear. Although some studies have reported no correlation between community structure and fertilization (Schaffer *et al.*, 2001; Piceno and Lovell, 2000), we found that a significant percentage of the community variation could be explained by nitrate (28.6%) and ammonium levels (16.1%), which is in accordance with the physiology of nitrogen fixation (common response to N availability). Moreover, agricultural practices are key determinants of bacterial community structures in soil (Patra *et al.*, 2006; Salles *et al.*, 2006; Wakelin *et al.*, 2009). Besides N, soil texture also represents a critical variable, as clay fractions in soils are important in forming microaggregates (Gupta and Roper 2010). This establishes microaerophilic or anaerobic conditions, providing more adequate environments for to oxygen-sensitive nitrogen fixation process. Even though we did not measure the rates of nitrogen fixation in our samples, we observed changes in community sizes and structures of the N fixers in response to soil structure. It has been shown that changes in N fixation rates may be a function of the diversity of the nitrogen fixing community (Hsu and Buckley, 2009).

Seasonal variation in the composition of the nitrogen-fixing community

In order to identify and characterize the members of the N-fixing communities in our samples, we have chosen pyrosequencing based on the *nif*H-gene. Considering the enormous bacterial diversity in the soil ecosystem, it is not unexpected that a high diversity of nitrogen-fixing microorganisms is also found (Duc *et al.*, 2009; Mao *et al.*, 2011). Although some studies have shown substantial stability of the diazotrophic assemblage over an annual cycle (Piceno *et al.*, 1999; Piceno *et al.*, 2000) based on PCR-DGGE, a deeper analysis of this community through pyrosequencing, such as performed by us, revealed a strong seasonal variation in the composition of the *nif*H gene sequences. Temporal variability in community composition of the N fixers is often reported in marine environments (Foster *et al.*, 2009; Kong *et al.*, 2011; Gobet *et al.*, 2012), but it has been rarely studied in terrestrial ecosystems (Mao *et al.*, 2011).

The sequences recovered from the clone libraries were affiliated with several major groups of bacteria, which changed greatly with sampling time. Soils B and D showed the lowest percentage of shared species between sampling times, indicating that large fractions of the nitrogen-fixing community in these soils are under constant replacement, with some populations disappearing completely and later on reappearing during the time period analyzed (Gobet *et al.*, 2012). This internal restructuring of the diazotrophic community has been previously reported for these sites (Pereira e Silva *et al.*, 2011), and it seems to occur to a higher extent in the sandy soils than in the clayey ones. The influence of soil

type on the composition of the N-fixing communities was supported by UniFrac analysis, confirming the results obtained by CCA analysis of DGGE patterns.

The dominance of *Alphaproteobacteria* (in specific *Bradyrhizobium*) across all soils and sampling times is not surprising, as it was observed previously not only in terrestrial (Smit *et al.*, 2001; Buckley and Schmidt, 2003), but also in marine environments (Gobet *et al.*, 2012). However, some groups followed seasonal fluctuations. For instance, the class *Gammaproteobacteria* mostly represented by *Methyloccocales*, *Pseudomonadales* and *Chromatiales*, increased significantly throughout the season. This season- dependent increase in the abundance of nitrogen-fixing *Gammaproteobacteria* towards the end of the season might reflect the copiotrophic behavior of the members of this group, which tend to be favored under nutrient-rich conditions (Zavarzin *et al.*, 1991; Ozgul *et al.*, 2011). It might be that in June and October more nutrients were available to soil diazotrophs than in April.

Cosmopolitan *nif*H species are identified as the species that are present across time and soil type. In this class, we found *Bradyrhizobium*, *Azospirilum*, *Methylocistis* in high abundance, as found elsewhere (Pereira e Silva *et al.*, 2011; Chowdhury *et al.*, 2009; Coelho *et al.*, 2008) . The high abundance of sequences affiliated with *Bradyrhizobium* is interesting as it is known as a symbiotic N-fixer, whereas there is increasingly understand that it also may have a key metabolic role as a soil saprophyte. The field we investigated are used for potato cropping and subjected to crop rotation with non-leguminous plants. It is very likely that the *Bradyrhizobium* types found are excellent survivors across all the diverse conditions applied in these farming systems. Moreover, they also abound in Canadian forest soils, as evidenced in a recent metagenomics based study. *Azospirillum*, on the other hand, is a genus of non-symbiotic (yet plant-associated) N-fixers that is found almost everywhere on Earth (Döbereiner and Pedrosa 1987; Huergo *et al.*, 2008). It is known to be associated with roots of grasses, cereals, food crops and soils (Peng *et al.*, 2006). We also detected the "core" species of each soil, as the species that were observed only in a particular soil, soil type and/or at a particular sampling time, which represents the types that are more sensitive to environmental variations (e.g. *Paenibacilus*, *Burkholderia* and *Celerinatantimonas* species). The low percentage of shared OTUs among soil suggests that each soil, with its own structure and characteristics, harbors a particular N-fixing community. Indeed, soil type has been reported as the primary driver of soil bacterial composition (Girvan *et al.*, 2003).

Overall, our results thus indicate an interaction between sampling time and environment with respect to affecting the evenness of the community, causing an enrichment of dominant OTUs in detriment of others at the beginning of the season. Roesch and co-workers (2010) came to a similar conclusion when analyzing the diazotrophic communities of soils from Brazil and Canada. The

authors found 11% of the community was shared between the sites, which were separated by up to 9,000 km and suggested that the free-living N-fixers were not cosmopolitan but rather selected by the environment. In the present study, three main environmental variables were identified as main effectors of the communities, i.e, ammonium, pH and clay content. Indeed, although the *nif*H based diversity was high in the clayey soils, a lower turnover rate through time was found. The effect of ammonia on the nitrogen fixation process has been recognized for a long time, as nitrogenase is commonly inhibited by the presence of NH_4^+ ions (Brotonegro, 1974; Houwaard, 1978; Christiansen-Weninger and van Veen, 1991).

The abundant *nif*H carrying bacterial groups are thought to be well adapted to their environment (Zhang *et al.*, 2006). However, we cannot discard the possibility that some rare species might also be relevant for biogeochemical cycling processes. The rare diazotrophic species also responded to environmental parameters, suggesting that the fluctuations on both abundant and rare microbial types are under the control of environmental factors. Also, the variations in physical and chemical properties between different soils might select for different species as the most dominant members as well as the rare ones. This structural shift might have an ecological importance, as reported by Hsu and Buckley (2009). Interestingly, some of the rare members increased greatly their abundance at some point in time, probably when the appropriate conditions were met, acting as a source.

Concluding, we are at the auge of understanding what drives the diazotrophic bacterial communities in soils at a very thorough level. The broad data set obtained in this study provides a fundament for future directed studies on individual performance, e.g. of the cosmopolitan (e.g. *Bradyrhizobium*) or specific (e.g. *Azoarcus*) *nif*H containing species.

Acknowledgments

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Table S1. Soil characteristics measured in this study.

Abiotic parameter	Sampling time	Soils			
		Buinen (B)	Drovendaal (D)	Kollumerward (K)	Grebbedijk (G)
pH	April	4.20	5.00	7.40	7.20
	June	4.60	5.10	7.40	7.00
	October	4.40	4.70	7.40	7.40
	Average	4.40	4.93	7.40	7.20
	C.V.	0.05	0.04	0.00	0.03
OM	April	4.90	2.60	6.60	6.40
(%)	June	3.50	3.30	3.30	4.30
	October	3.60	2.60	2.70	5.50
	Average	4.00	2.83	4.20	5.40
	C.V.	0.20	0.14	0.50	0.20
Water content	April	13.80	9.50	16.70	18.80
(%)	June	7.50	16.00	19.70	20.40
	October	12.30	9.40	21.50	19.60
	Average	11.20	11.63	19.30	19.60
	C.V.	0.29	0.33	0.13	0.04
$N\text{-}NO_3^-$	April	24.50	67.90	43.60	59.00
(mg/kg)	June	103.60	68.50	14.80	12.20
	October	13.40	45.20	15.40	18.60
	Average	47.17	60.53	24.60	29.93
	C.V.	1.04	0.22	0.67	0.85
$N\text{-}NH_4^+$	April	10.40	18.40	9.80	19.80
(mg/kg)	June	12.80	15.30	6.60	21.20
	October	4.50	3.20	8.80	4.40
	Average	9.23	12.30	8.40	15.13
	C.V.	0.46	0.65	0.19	0.62

OM = organic matter; $N\text{-}NO_3^-$ = nitrate and $N\text{-}NH_4^+$ = ammonium. Numbers are average of three replicates

Table S2. PCR and cycling conditions for PCR-DGGE analysis, real time quantification and pyrosequencing of *nif*H gene.

	PCR mixture	Thermal conditions
Primers DGGE (5′-3′)		
FPGH19 (TACGGCAARGGTGGNATHG) PolR (ATSGCCATCATYTCRCCGGA)	0.20mM dNTPs, 1x buffer (Roche), 0.01mg BSA (20mg/ml), 0.5µM each primer, 0.5U Taq polymerase (Roche)	94°C, 5 min 94°C 60s, 56°C 1 min, 72°C 2min 30 cycles final ext. of 72ºC 30 min
PolF-GC * (TGCGAYCCSAARGCBGACTC) AQER (GCCATCCATCTGTATGTCCA)	0.25mM dNTPs, 1x buffer (Roche), 0.01mg BSA (20mg/ml), 0.5µM each primer, 0.8U Taq polymerase (Roche)	94°C, 5 min 94°C 60s, 48°C 1 min, 72°C 2min 30 cycles final ext. of 72ºC 30 min
Primers qPCR (5′-3′)		
FPGH19 (TACGGCAARGGTGGNATHG) *PolR* (ATSGCCATCATYTCRCCGGA)	12.5µl Power Sybr Green PCR Master mix, 0.5ul BSA (20mg/ml), 0.25µM each primer and 2ul DNA template	95°C 10 min, 1 cycle 94°C for 60s, 55°C for 27s, 72°C for 60s, 40 cycle
Primers pyrosequencing (5′- 3′)		
PolF (TGCGAYCCSAARGCBGACTC) *PolR* (ATSGCCATCATYTCRCCGGA)	0.20mM dNTPs, 1x buffer (Roche), 0.03mg BSA (20mg/ml), 0.5µM each primer, 0.25U FastStart High Fidelity PCR System, 50ng template DNA	95°C, 5 min 94°C 1 min, 48°C 1 min, 72°C 1min 35 cycles final ext. of 72ºC 10 min
RoeschF (ACCCGCCTGATCCTGCACGC CAAGG) *RoeschR* (ACGATGTAGATTTCCTGGGC CTTGTT)	0.20mM dNTPs, 1x buffer (Roche), 0.03mg BSA (20mg/ml), 0.5µM each primer, 0.25U FastStart High Fidelity PCR System, 50ng template DNA	95°C, 5 min 94°C 45s, 50°C 45s, 72°C 45s 20 cycles final ext. of 72ºC 10 min

*GC clamp according to Muyzer *et al.*, 1999.

Table S3. Richness estimates and diversity indices for forward and reverse amplicon libraries at 90% similarity cutoff.

Library	NS[a]	OTUs[b]	Estimated OTU richness		Shannon[c]	ESC[d]
			Chao1	ACE		
Forward						
B_Ap	1921	40	48.25 (42.16; 71.48)	53.53 (44.54; 80.31)	2.09 (2.03; 2.15)	0.95
D_Ap	2981	87	135.33 (105.92; 210.49)	130.69 (107.37; 108.69)	2.81 (2.75; 2.86)	0.93
K_Ap	3530	80	119 (94.76; 183.05)	123.4 (99.41; 177.03)	2.53 (2.47; 2.58)	0.91
G_Ap	3313	71	102.67 (81.39; 167.45)	99.46 (82.48; 141.56)	2.66 (2.61; 2.71)	0.92
B_Ju	2071	44	65 (50.07; 116.68)	59.55 (49.57; 87.45)	1.98 (1.92; 2.04)	0.95
D_Ju	2779	65	78.6 (69.29; 108.09)	89.17 (74.59; 125.92)	2.66 (2.61; 2.72)	0.93
K_Ju	5141	118	224 (168.11; 342.25)	256.19 (192.21; 375.32)	2.83 (2.79; 2.88)	0.88
G_Ju	5892	110	196.67 (146.63; 315.03)	174.71 (142.76; 223.78)	2.93 (2.89; 2.97)	0.86
B_Oc	2266	63	110.25 (80.87; 187.87)	134.79 (95.36; 222.26)	2.58 (2.53; 2.64)	0.95
D_Oc	3671	120	183.58 (150.01; 254.72)	193.92 (160.06; 256.39)	2.98 (2.94; 3.03)	0.91
K_Oc	4171	107	164 (131.98; 237.07)	173 (141.24; 237.01)	2.94 (2.89; 2.99)	0.90
G_Oc	3669	74	108.5 (86.41; 169.855)	106.57 (88.35; 147.91)	2.47 (2.43; 2.52)	0.91
Reverse						
B_Ap	1916	39	124.50 (63.95; 131.96)	112.53 (62.16; 272.43)	2.16 (2.11; 2.22)	0.96
D_Ap	3346	80	122.16 (94.52; 202.49)	109.53 (92.31; 150.81)	2.88 (2.83; 2.93)	0.93
K_Ap	3791	82	136 (102.11; 227.01)	123.41 (100.33; 175.56)	2.82 (2.78; 2.86)	0.92
G_Ap	3311	67	94.14 (76.04; 148.51)	95.25 (78.69; 135.26)	2.49 (2.45; 2.54)	0.93
B_Ju	2317	37	53.5 (41.25; 100.95)	55.29 (42.96; 93.12)	2.09 (2.04; 2.15)	0.95
D_Ju	3416	72	91 (78.30; 129.34)	91.40 (80.04; 118.82)	2.62 (2.57; 2.67)	0.92
K_Ju	5372	94	146.8 (115.49; 223.69)	172.97 (131.78; 259.11)	2.99 (2.96; 3.03)	0.88
G_Ju	6328	101	157.1 (124.04; 237.63)	156.32 (127.74; 215.48)	2.84 (2.80; 2.88)	0.86
B_Oc	2422	40	85.5 (52.08; 211.299)	65.88 (48.46; 119.12)	2.34 (2.29; 2.39)	0.95
D_Oc	3845	82	183.5 (117.14; 375.17)	138.76 (106.86; 211.57)	2.91 (2.87; 2.96)	0.91
K_Oc	4944	101	146.77 (120.43; 208.8)	159.08 (129.79; 218.16)	2.99 (2.95; 3.03)	0.89
G_Oc	3760	57	109.5 (73.71; 221.96)	99.61 (73.62; 166.27)	2.37 (2.33; 2.420	0.92

[a]Number of sequences in each library.
[b]Calculated with DOTUR at the 10% distance level.
[c]Shannon diversity index calculated using DOTUR (10%).
[d]Estimated sample coverage: Cx = 1 - (Nx/n), where Nx is the number of unique sequences and n is the total number of sequences.
Values in brackets are 95% confidence intervals as calculated by DOTUR.

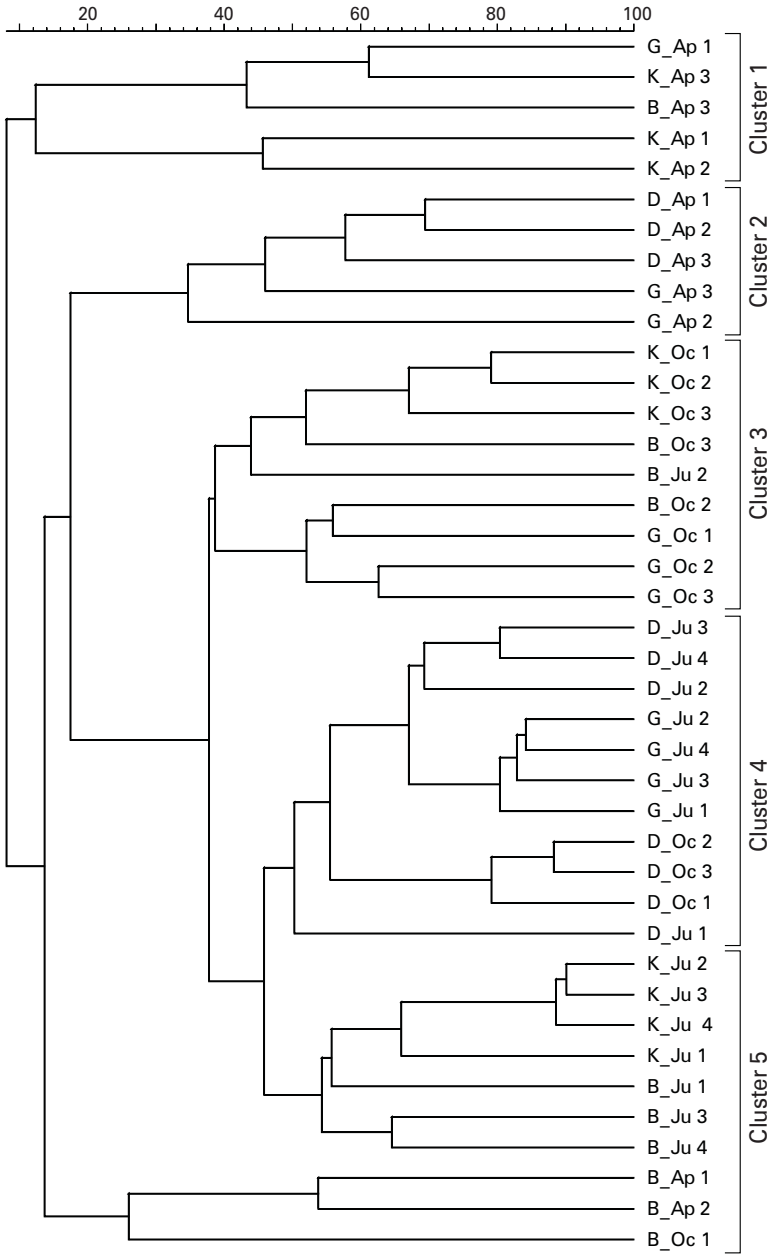

Figure S1. Unweighted pair group method with average linkages dendograms of diazotrophic community associated with four soils.

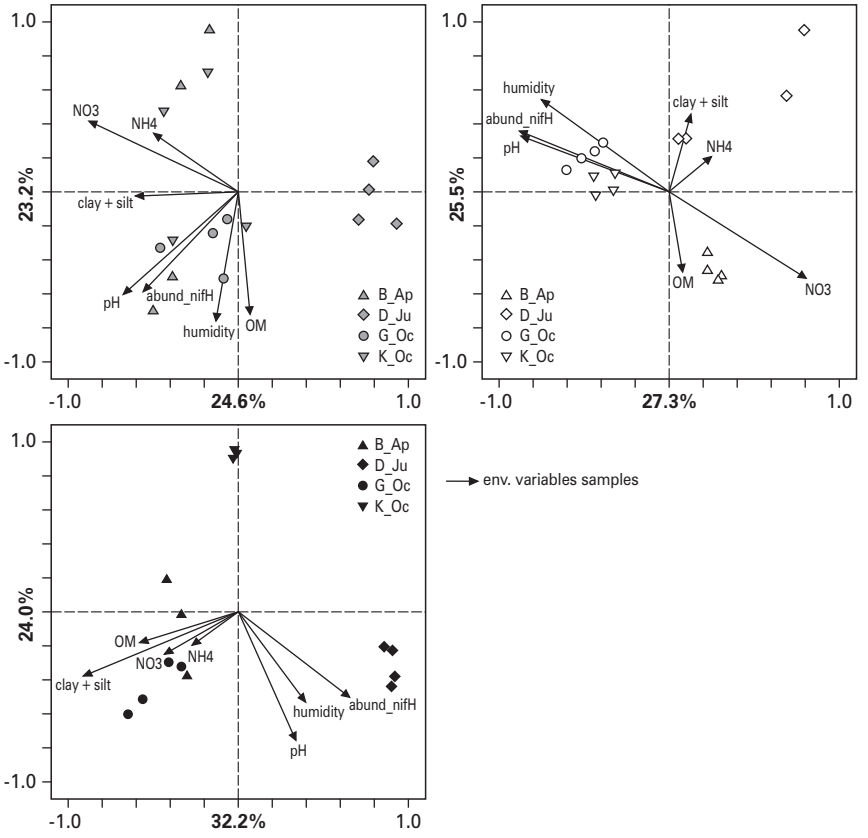

Figure S2. Changes in the structure of *nif*H-gene across different soils at each sampling time, and the influence of environmental parameters, as revealed by Canonical correspondence analysis (CCA). The numbers in each axis shows the percentage of total variation explained. The length of the corresponding arrows indicated the relative importance of the geochemical factor in explaining the variation in microbial profiles. Soil samples were analyzed in four replicates at each sampling time. B, Buinen; D, Droevendaal; K, Kollumerwaard and G, Grebbedijk.

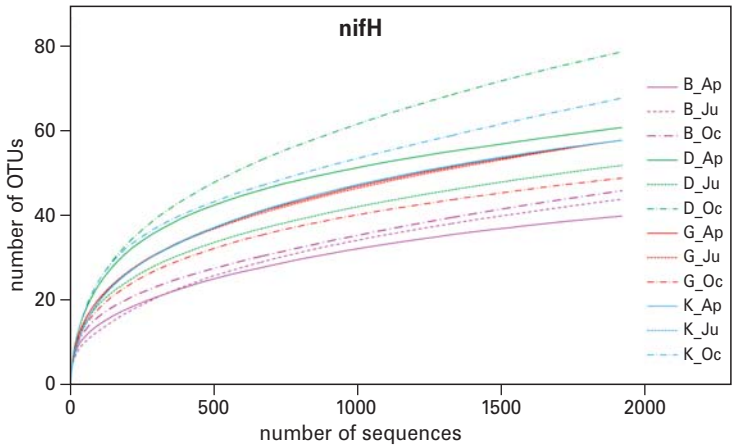

Figure S3. Rarefaction analysis of the diversities of *nif*H gene in the four soils and three sampling times after resampling of the sequences to the same depth. The OTUs were classified at 90% similarity cutoff based on amino acid sequences. B, Buinen; D, Droevendaal; K, Kollumerwaard and G, Grebbedijk; Ap, April; Ju, June; Oc, October.

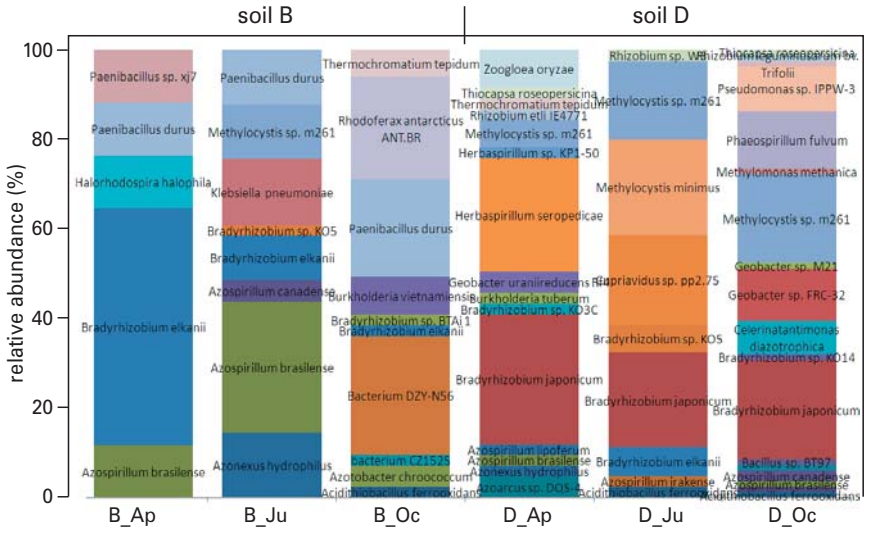

Figure S4. Taxonomic classification of the less abundant *nif*H-gene sequences associated with the two sandy agricultural soils and at three sampling times, April, June and October. Multi-colored charts at the legend are shown for each sample correspondingly.

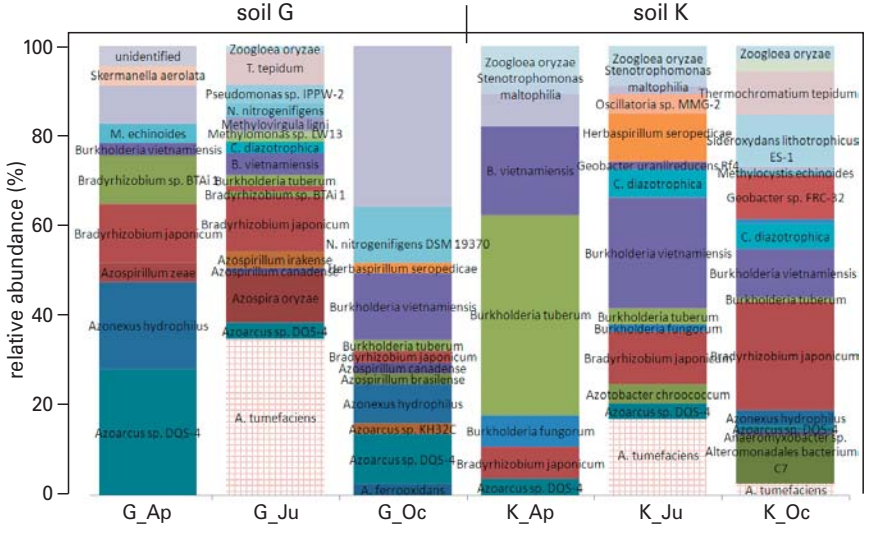

Figure S5. Taxonomic classification of the less abundant *nif*H-gene sequences associated with the two clayey agricultural soils and at three sampling times, April, June and October. Multi-colored charts at the legend are shown for each sample correspondingly.

Chapter 6

Differential response of archaeal ammonia oxidizing communities to soil environmental parameters

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Abstract

The oxidation of ammonia is the rate-limiting step in the biological nitrification process, and it can be carried out not only by ammonia oxidizing bacteria (AOB), but also by ammonia oxidizing archaea (AOA). It has been suggested that some AOA are adapted to high pH environments and others to those with low pH. However, how these different groups respond to other soil parameters and the extent to which they matter to nitrification has remained unanswered. Here, we determined the abundance, diversity and composition of AOA in four agricultural soils during one growth season, i.e. 2010. We also measured relevant soil parameters and potential nitrification activity (NEA). NEA rates, as well as *amo*A gene copy abundances, were significantly lower in soils with low pH and low clay content (denoted B and D) and higher in soils with high pH and high clay content (G and K). We performed bar-coded pyrosequencing based on *amo*A. In total, 81,029 reads were obtained, yielding 695 – 2140 amino acid residues per sample. The diversity estimates as well as the OTU numbers observed were higher in the soils with lower pH (B and D), compared to those with higher pH (K and G). Clustering of the sequence reads at 90% similarity cutoff resulted in 50 clusters with more than 10 sequences each. These were spread over two known archaeal clusters, the Soil/Sediment cluster (Cluster I, containing OTUs from acid and alkaline soils), and the Sediment/Soil cluster (Cluster II, containing only OTUs from alkaline soils). These clusters were analyzed separately and tested against the environmental parameters using the BEST test. Whereas Cluster 1 was mainly correlated with soil pH, nitrate and clay content, Cluster 2 was influenced by pH, clay content and OM. The relationships between NEA and these clusters were also distinct, with Cluster 2 showing higher correlations than Cluster 1. This study suggests that the archaeal clusters commonly found in soils are well adapted to specific pH ranges and respond differently to other soil parameters, which has consequences for the nitrification process.

Introduction

Nitrogen is a key nutrient for all organisms (Le Bauer and Treseder, 2008), as it is a critical component of proteins, DNA and RNA, fundamental to the biochemical processes that define life (Francis *et al.*, 2007). Therefore, nitrogen transformations are important, including the microbially-mediated process of nitrogen fixation, denitrification and nitrification (Bernal *et al.*, 2009; Maeda *et al.*, 2011). Nitrification is a two-step process. The oxidation of ammonia via hydroxyl amine to nitrite is the first and rate-limiting step, which is followed by the conversion of nitrite to nitrate (Kowalchuk and Stephen 2001). In the former step, ammonia monooxygenase (encoded by the so-called *amo* gene) is the key functional enzyme. This step was initially thought to be uniquely performed by ammonia oxidizing bacteria (AOB) from β- and γ-subclasses of the Proteobacteria. However, evidence that also archaea can be involved in the oxidation of ammonia (AOA) (Treusch et al 2005; Könneke et al 2005) has challenged that paradigm. Initially, the AOA were classified as mesophilic Crenarchaeota (Treusch *et al.*, 2005). Later on, phylogenetic analysis of ribosomal and protein-encoding genes led to the separation of the AOA into a new *phylum*, Thaumarchaeota (Brochier-Armanet *et al.*, 2008). Since then, AOA have been observed in multiple environments (Wutcher *et al.*, 2006; Nicol *et al.*, 2008; Di *et al.*, 2010; Gubry-Rangin *et al.*, 2010, 2011). In particular, they were found to dominate in several soils (Leininger *et al.*, 2006) when compared to AOB.

Nitrification is directly related to to leaching of nitrate leaching from soils (Kowalchuk and Stephen 2001). Thus, the autotrophic nitrification is linked to a main pathway of nitrous oxide (greenhouse gas) production in the soil environment (Kowalchuk and Stephen 2001; Colliver and Stephenson 2000). However, despite their environmental importance, there is still uncertainty and controversy regarding the distribution of ammonia oxidizers in soil as well as their tolerances and responses to the environment (Wankel *et al.*, 2010). Moreover, it has remained unclear whether nitrification is mainly driven by archaeal or bacterial ammonia oxidizers and under which conditions this occurs. Some studies have proposed soil pH (He *et al.*, 2007; Hallin et al 2009; Nicol *et al.*, 2008, Erguder *et al.*, 2009; Gubry-Rangin *et al.*, 2012) as a key determinant of the ammonia oxidizing communities. Temperature (Christman *et al.*, 2011; Tourna *et al.*, 2011), N availability and moisture (Jia and Conrad 2009; Hallin *et al.*, 2009; He *et al.*, 2007), next to soil management techniques (Berg and Rosswall, 1987; Le Roux *et al.* 2008), have also been shown to influence the soil nitrifiers. Furthermore, it was recently suggested that some ammonia oxidizing archaea (AOA) are specialized to environments with high pH and others to low pH (Gubry-Rangin *et al.*, 2012). However, how these different pH-sensitive groups respond to other soil parameters and the extent to which they drive nitrification under field conditions remain unanswered.

Considering the great important of nitrification and the perceived dominance of archaeal ammonia oxidizers (Pereira e Silva *et al.*, 2012), in the present study we determined the size, diversity and composition of AOA from four agricultural soils at three times over one growing season in 2010. Community sizes were studied by quantifying the archaeal *amo*A genes, whereas diversity and composition were determined by pyrosequencing of the archaeal *amo*A genes. We also measured relevant chemical soil parameters and potential nitrification activity. Our goal was to establish links between nitrification, species diversity, abundance and composition of AOA in agricultural soil under a long history of N fertilization. Moreover, we aimed at gathering information on whether and to what extent the AOA cluster respond to common soil variables, and how this relates to nitrification rates.

Material and Methods

Sampling sites

Four soils, named here B (Buinen), D (Droevendaal), G (Grebbedijk) and K (Kollummerwaard), from different sites in the Netherlands were sampled three times in 2010, April (after seedling), June (before flowering) and October (senescence stage). The fields are used for potato cropping and are under agricultural rotation regime with non-leguminous crops. Information on land-use and location is available in Table 6.1. The soils were chosen to represent different soil types (clay vs. sand) and present different chemical properties (Table 6.1 and Table S1). Bulk soil samples (4 replicates per soil; 0.5kg per replicate) were collected in plastic bags and thoroughly homogenized before further processing in the lab. A 100-g subsample was used for measuring ammonia oxidizing enzyme activity, molecular biology and soil chemical properties.

Soil chemical analysis and activity measurements

The soil pH was measured after shaking a soil/water (1:2, w:v) suspension for 30 min (Hanna Instruments BV, IJsselstein, The Netherlands). Gravimetric soil moisture conents were determined comparison of fresh and dried (105ºC; 24h) weight of samples. Organic matter (OM) content is calculated as the difference between the initial and final sample weights of dried soil measured after 4 hours at 550ºC. Nitrate ($N\text{-}NO_3^-$) and ammonium ($N\text{-}NH_4^+$) were determined in $CaCl_2$ extracts by a colorimetric method using the commercial kits Nanocolor Nitrat50 (detection limit, 0.3 mg N kg^{-1} dry weight, Macherey-Nagel, Germany) and Ammonium3 (detection limit, 0.04 mg N kg^{-1} dry weight; Macherey-Nagel, Germany) according to Töwe *et al.* (2010) .

Nitrifying enzyme activity protocol was adapted from Dassonville *et al.*, 2011. Potential Ammonia–oxidizing enzyme activity (NEA) was determined by

using soil suspensions in the presence of non-limiting ammonium and ambient atmospheric O_2 concentration. Plasma flasks (150 mL) containing fresh soil (equivalent to 3 g ovendried soil) and 30 mL of $(NH_4)_2SO_4$ (1.25 mg N. L^{-1}) were incubated on a rotary shaker (180 rpm, 28°C). The production of nitrate and nitrite during 10 hours of incubation was monitored by periodic (2h, 4h , 6h, 8h and 10h) withdrawal of 2 ml samples by means of a syringe. The samples were filtered at 0.20 µm to remove soil particles and microorganisms. Samples were stored at -18°C prior to analysis. The filtrate was analyzed for nitrate and nitrite content using an ionic chromatography (DX120, Dionex, Salt Lake City, USA) equipped with a 4 x 250 mm column (IonPac AS9 HC). The NEA was calculated from the slope of the linear regression curve of nitrate plus nitrite production versus time.

DNA extraction

DNA was extracted from 0.5 g soil using the FastDNA SPIN Kit for Soil (MP Biomedicals) according to manufacturer's protocol. Extracted DNA was then precipitated and concentrated with cold ethanol to remove impurities. DNA concentrations were determined using the Quant-iT™ PicoGreen® dsDNA Reagent (Invitrogen, Darmstadt, Germany) according to the manufacturer's protocol using a spectrofluorometer (Spectramax Gemini, Molecular Devices GmbH, Germany). The quality of extracted DNA was estimated by running on agarose gel based on the degree of DNA shearing (average molecular size) as well as the amounts of co-extracted compounds.

Table 6.1. Sample locations, environmental and biological data.

Sampling Location	Buinen (B)	Droevendaal (D)	Kollummerwaard (K)	Grebbedijk (G)
Soil type	Sandy loam	Sandy loam	Clayey	Clayey
Land use	Agricultural	Agricultural	Agricultural	Agricultural
Sampling coordinates	52°55′386″N 006°49′217″S	51°59′551″N 005°39′608″S	53°19′507″N 006°16′351″S	51°57′349″N 005°38′086″S
pH	4.40±0.02	4.93±0.02	7.40±0.05	7.20±0.02
N-NO_3^- (mg/kg)	47.51±0.37	60.53±1.66	24.60±1.72	29.93±1.15
N-NH_4^+ (mg/kg)	9.23±0.74	14.18±1.41	8.40±0.75	15.13±2.35
OM (%)	3.96±0.72	2.91±0.24	4.24±0.14	5.47±0.22
Water content (%)	11.20±0.58	11.63±1.62	19.30±1.40	19.60±1.86
NEA (µgN.h^{-1}.gdw^{-1})	0.23±0.03	0.14±0.02	0.98±0.18	1.67±0.09
*amo*A-gene Abund. (Log_{10} gdw^{-1})	6.08±0.12	6.01±0.15	6.72±0.08	7.00±0.07

Values of environmental and biological data are average of each soil across the three sampling times (mean ± sd).

Real-time quantitative PCR (qPCR)

The abundances of archaeal *amo*A genes in all the soils samples were quantified using real-time PCR. For AOA, primers crenamo23F (Tourna *et al.*, 2008) and crenamo616r (Nicol *et al.*, 2008) were used obtaining fragments of 624 bp. Cycling programs and primer sequences are detailed in Table S2. Standard curves were generated from serial dilutions of plasmid containing cloned archaeal *amo*A gene, from 10^6 to 10^2 gene copy numbers/µl. Absolute quantification was carried out twice from each of the four soil replicates on the ABI Prism 7300 Cycler (Applied Biosystems, Germany). The specificity of the amplification products was confirmed by melting-curve analysis, and the expected sizes of the amplified fragments were checked in a 1.5% agarose gel stained with ethidium bromide. Possible inhibitory effects of co-extracted humid compounds were checked by spiking standard concentrations with serial dilutions of soil samples. No severe inhibition was observed at the working dilutions.

Barcoded pyrosequencing and bioinformatic analysis

Diversity of archaeal ammonia oxidizers in the four agricultural soils was investigated by barcoded pyrosequencing approach. The total community DNA was amplified with *amo*A gene-specific primers crenamo23F (Tourna *et al.*, 2008) and crenamo616r (Nicol *et al.*, 2008) in a nested approach using the FastStart High Fidelity PCR system and PCR Nucleotide Mix (Roche Diagnostics GmbH, Mannheim, Germany). PCR conditions and primer sequences are found in Table S2. Triplicate PCR amplifications were performed on each soil DNA template and pooled. Primer dimers were removed by electrophoresis of PCR products on agarose gel, excision, and purification using Qiaquick PCR purification Kit (Qiagen). For 454 pyrosequencing of samples, adapters and sample-specific tags were added using custom primers in an additional PCR amplification of 20 cycles using the same PCR conditions. Amplicons were further purified with AMpure beads (Beckman Coulter) and pooled in an equimolar ratio as specified by Roche. Sequencing from 5′ (forward) and 3′ (reverse) ends of amplicons was performed. Emulsion PCR, emulsion breaking of DNA-enriched beads, and sequencing runs of the amplicon pools were performed on a second-generation pyrosequencer (454 GS FLX Titanium; Roche) using titanium reagents and titanium procedures as recommended by the manufacturer. The 454-pyrosequencing data have been deposited in the National Center for Biotechnology Information (NCBI) under accession number xxxxxx to yyyyyy.

Quality filtering of the pyrosequencing reads was performed using the automatic amplicon pipeline of the GS Run Processor (Roche) to remove failed and low-quality reads from raw data. Amplicon libraries of the ammonia monooxigenase gene (*amo*A) were explored using the FunGene Pipeline of RDP server (http://fungene.cme.msu/edu/FunGenePipeline) using the default settings.

Primer sequences were trimmed and reads of low quality and shorter than 400 bp were removed. Filtered nucleotide sequences were translated into amino acid. All subsequent analyses were done on amino acid sequences. By targeting a protein-coding gene, frame-shifts errors caused by insertions or deletions of bases, can be identified (Huse *et al.*, 2007). Sequences were then visually inspected and sequences having in-frame stop codon(s) were removed. The amino acid sequences were aligned by MUSCLE 3.8 (Gouy *et al.*, 2010).

Operational taxonomic units (OTUs) were then classified and rarefaction curves were constructed with DOTUR (Schloss and Handelsman 2005) using 90% amino acid sequence similarity cutoff (Palmer *et al.*, 2009; Mao *et al.*, 2011). Richness estimates and diversity indices were calculated for the total number of sequences as well as for the subsets normalized to the same number of sequences by the Perl script daisychopper.pl (available at http://www.genomics.ceh.ac.uk/ GeneSwytch/Tools.html). Phylogenetic analysis was performed for clustered sequences at 90% similarity using CD-HIT (Li and Godzik, 2006). The representative sequences (of more than 10 sequences) were used to build neighbor-joining trees in MEGA5 (Tamura *et al.*, 2011). Normalized weighted UniFrac significance (Lozupone *et al.*, 2006) was calculated to evaluate differences between the *amo*A-gene communities and for clustering analyses based on the phylogenetic trees obtained in Mega5.

Data analysis

AOA community composition data obtained from pyrosequencing were used to construct Bray Curtis similarity matrices and analyzed in PRIMER-E software package (version 6, PRIMER-E Ltd, Plymouth, UK; Clarke and Gorley, 2006). Non-metric multidimensional scaling (MDS) were used to analyze similarity/dissimilarity of genetic structures among soil samples based on 5000 iteractions and maximally three dimensions. The correlation between biological data and soil chemical parameters were tested using Global BEST test in Primer-E software (with Spearman Coefficient and 5000 permutations), which selects environmental variables "best explaining" community pattern, by maximizing a rank correlation between their respective resemblance matrices. Correlations between NEA and AOA community structure were tested using the RELATE analysis, a non-parametric form of Mantel test, implemented in PRIMER-E software. More specifically, a rank correlation coefficient (here Spearman coefficient) and significance level (obtained by a permutation test using 5000 permutations) were computed to quantify the correlation between the rank similarity matrices obtained for activity and genetic structure. Differences in soil chemical parameters and *amo*A gene abundances in the different soils over time were estimated with independent t-Tests.

Results

Fluctuations in soil chemical properties and nitrifying activity (NEA)

Soil pH, nitrate, ammonium and organic matter levels were determined in triplicate across all samples. Considering all soils, pH and OM values were significantly higher ($P < 0.05$) in the clayey soils K and G than in the sandy soils B and D (Table 6.1). In contrast, levels of nitrate were higher in soils B and D (47.17 ± 0.37 and 60.53 ±1.66, respectively) than in soils K and G (29.93 ± 1.15 and 24.60 ± 1.72, respectively), with the first showing significantly higher values in June than in April and October, whereas the latter showed significantly depressed nitrate levels in the same period. Levels of ammonium also varied over the whole period. Low values were observed for all soils in October (on average 5.47 mg/kg ± 0.66) whereas higher levels were detected in April and June (on average, 14.63 mg/kg ± 2.66 and 14.01 mg/kg ± 0.61, respectively). Individual values for each soil at each sampling time can be found in Table S1. Variations in NEA over time were observed in all soils. Overall, the lowest nitrification rates were observed in soil D from June (0.1485 $\mu gN.h^{-1}.gdw^{-1}$ ± 0.02), whereas the highest rates were measured in soil G from April (0.94 $\mu gN.h^{-1}.gdw^{-1}$ ± 0.05) (Fig. 6.1). On average and per time of sampling, lower rates were observed in the June samples (0.63 $\mu gN.h^{-1}.gdw^{-1}$ ± 0.07), whereas higher rates were detected in the April (0.82 $\mu gN.h^{-1}.gdw^{-1}$ ± 0.05) and October samples (0.81 $\mu gN.h^{-1}.gdw^{-1}$ ± 0.11).

Fluctuations in *amo*A gene abundance and responses to soil variables

The of *amo*A gene abundances fluctuated from 5.06×10^5 to 2.53×10^7 gene copies gdw^{-1} soil over the growing season, being significantly higher ($P < 0.05$) in June (1.06×10^7 gene copies gdw^{-1}) compared to April (2.14×10^6 gene copies gdw^{-1}) and October (4.66×10^6 gene copies gdw^{-1}) (Fig. 6.2). Analysis per soil revealed that the clayey soils K and G had significantly higher *amo*A gene abundance at all sampling times (9.65×10^6 gene copies gdw^{-1} on average) than the sandy soils B and D (1.51×10^6 gene copies gdw^{-1} on average). Pearson's product-moment correlations were calculated to test the influence of soil variables on *amo*A gene abundances. Different parameters were found to correlate with gene abundance at each sampling time. Overall, the main determinants of *amo*A gene abundance were nitrate (-0.478, P = 0.001), pH (0.716, P = 0.000), moisture (0.554, P = 0.000) and clay content (0.744, P = 0.000). Strong correlations were observed between the mean values of nitrifying activity and AOA abundance in April, June and October (Fig. S1).

Dynamics of the AOA community composition

To further understand the changes in the AOA community compositions in the four agricultural fields, the *amo*A gene was deep-sequenced using pyro-

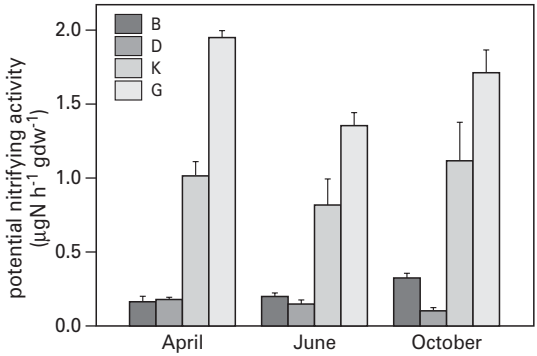

Figure 6.1. Potential nitrifying enzyme activity (NEA) measured in the four soils from April to October 2010. Soil names: B, Buinen; D, Droevendaal; K, Kollumerwaard; and G, Grebbedijk. Bars are standard errors (n = 4).

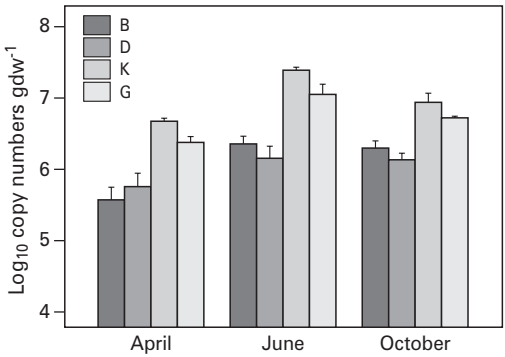

Figure 6.2. Real time PCR quantification of archaeal (AOA) *amo*A gene across two sandy (B and D) and two clayey (K and G) soils in April, June and October of 2010. Bars are standard errors (n = 4).

sequencing, where forward and reverse reads were analyzed separately. In total, 81,029 reads were obtained. Per sample, the translated amino acid sequences ranged from about 695 to 2140, 150 amino acids in length. This corresponded to 21 – 50 OTUs (defined at the 10% sequence difference cut-off). To allow for comparisons of diversity and richness among samples, the dataset was randomly resampled to the same sequencing depth (695 sequences per treatment for the forward and 742 for the reverse library), yielding an adjusted total number of OTUs between 7 and 32 (Table 6.2). The total numbers and diversity estimates are described in Table S3. From the forward libraries, we observed that, on average, the diversity estimates as well as the number of OTUs were higher in the soils with lower pH (B and D; pH between 4.4- to 5.0),

compared to those with higher pH (soil K and G; pH between 7.2 to 7.4), and this difference was significant ($P < 0.05$) (Table 6.2).These results were not significant in the reverse libraries. In the reverse libraries, we observed that the Shannon diversity indices were higher for the sandy soils (B and D) ($P < 0.05$) than for the clayey ones (soils K and G). We also observed that the diversity tended to be higher at the beginning of the season (April/ June) as compared to that at the end of the season (October). Rarefaction analysis of the *amo*A libraries resulted in distinct saturation profiles (Fig. S2). Libraries from soils B and D yielded still rising curves, whereas those from soil G and K were close to reaching the horizontal plateau (indicating saturation).

Clustering of the sequence reads using the 90% similarity cutoff level resulted in a total of 232 OTUs, from which 182 OTUs (1.62% of the dataset) were low in abundance (represented by less than 10 sequences). These sequences were removed from further analysis. The remaining sequences (19983) yielded 50 OTUs and corresponded to ca. 98.4% of the dataset. Collectively, twenty-three OTUs comprised ca. 90% (18,454 sequence reads) of the total libraries. In each soil, only around 25% of the OTUs were shared across all times. Strikingly, not a single OTU of the 50 detected was shared between all soils and sampling times. Moreover, only one OTU of 50 was shared between all soils in April (OTU 30),

Table 6.2. Richness estimates and diversity indices for reverse amplicon libraries at 90% similarity cutoff after random resampling of sequences to the same depth (1921 sequences).

Library Forward	OTUs[a]	Estimated OTU richness Chao1	ACE	Shannon[b]
B_Ap	21	26 (21.86; 49.91)	24.96 (21.855; 39.34)	1.38 (1.29; 1.48)
D_Ap	24	33 (26.13; 62.02)	39.85 (28.86; 75.62)	1.14 (1.04; 1.25)
K_Ap	25	51 (31.82; 124.18)	49.62 (32.70; 103.72)	1.27 (1.18; 1.36)
G_Ap	16	30 (18.92; 83.11)	34.07 (20.17; 94.37)	1.08 (0.99; 1.17)
B_Ju	26	32 (27.33; 53.10)	35.77 (28.84; 59.61)	1.23 (1.12; 1.33)
D_Ju	23	30 (24.34; 59.54)	27.64 (24.08; 42.87)	1.68 (1.61; 1.76)
K_Ju	19	26.2 (20.61; 51.37)	39.98 (24.89; 93.75)	0.56 (0.46; 0.66)
G_Ju	24	30.43 (25.51; 51.30)	37.01 (27.95; 66.80)	0.81 (0.71; 0.92)
B_Oc	23	34.25 (25.65; 70.72)	37.55 (27.15; 74.01)	1.42 (1.32; 1.51)
D_Oc	32	40.67 (34.37; 63.66)	49.95 (38.18; 84.15)	1.90 (1.81; 1.99)
K_Oc	14	21.5 (15.32; 56.530	21.86 (15.66; 51.09)	0.59 (0.51; 0.69)
G_Oc	23	30.2 (24.60; 55.37)	30.65 (25.01; 52.02)	1.10 (1.00; 1.21)

[a]Calculated with DOTUR at the 10% distance level.
[b]Shannon diversity index calculated using DOTUR (10%).
Values in brackets are 95% confidence intervals as calculated by DOTUR.

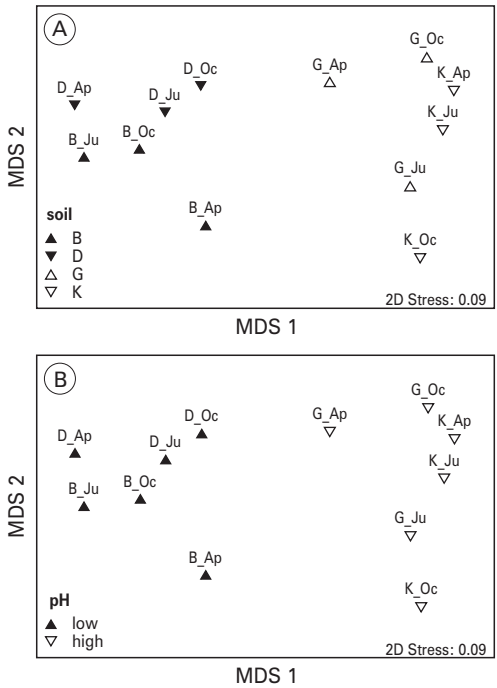

Figure 6.3. Non metric multidimensional scaling (MDS) based on OTU abundance of AOA sequences (A) and separated per soil pH (B). B, Buinen; D, Droevendaal; K, Kollumerwaard; and G, Grebbedijk; Ap, April; Ju, June and Oc, October.

three in June (OTUs 39, 43 and 51) and three in October (OTUs 40, 43 and 51) (Table S4 and Fig. S3). However, and again surprisingly, very few OTUs were site-specific (Table S4). However, several OTUs might be considered to be soil type specific, with seventeen (32.7% of the dataset) being unique to the sandy soils (B and D) and eight (15.38% of the dataset) to the clayey soils (G and K) (Table S4). To visualize the temporal dynamics of the communities at the level of clusters of OTUs, the 50 OTU types were used to compute a Bray-Curtis similarity matrix that was ordinated into two dimensions by NMDS (Fig. 6.3). Samples were primarily grouped by soil type, and secondarily by sampling time, but the latter effect was not consistent across all soils (Fig. S4)

Correlation of AOA community compositions with soil parameters

The consolidated 50 OTUs were used to construct phylogenetic trees, into which representative sequences from GenBank were integrated as well (Fig. S5). This phylogenetic analysis of the archaeal *amo*A fragments revealed relatedness of all clones mainly to sequences of uncultured crenarcheaeota that had been obtained in earlier environmental studies (Nicol *et al.*, 2008; Tourna *et al.*,

2008; Yao *et al*., 2011). Considering the whole dataset, soil pH, nitrate and OM levels explained a great part of the variation in AOA community composition (R = 0.753, P = 0.001). At the phylogenetic resolution investigated, most of the sequences (*ca*. 67%) clustered in the previously defined (Nicol *et al.*, 2008) archaeal Soil/Sediment cluster (Cluster I) and the remaining (33%) clustered in Sediment/Soil cluster (Cluster II). None of the OTUs grouped within the Marine cluster (Cluster III) (Fig. S5). We arbitrarily classified these three cluster based on presence of sequences from only acidic soil (pH ≤ 5.0), only alkaline soils (pH ≥ 7.0), and from both environments. Cluster I was classified as acido-alkalinophilic, with representatives from soil with low(4.5-5.0) and high pH (7.2-7.4). Cluster II was totally classified as acidophilic (Fig. S5). These specific groups of OTUs, acidic, acidic-alkalinophilic and alkalinophilic, were selected and used separately to construct Bray Curtis similarity matrices, which were tested separately against the environmental parameters using the Bioenv procedure in the BEST test (Primer-E software). The BEST test showed that these groups of sequences responded to different soil parameters to different extents (Table 6.3). The acidophilic group was positively correlated with soil pH, organic matter (OM) and clay content. The acido-alkalinophilic group correlated mainly with soil pH, nitrate and clay content, and the group of alkalinophic sequences was mostly correlated with soil pH, clay content and OM (Table 6.3). Nitrogen availability (nitrate and ammonium) was correlated to the acido-alkali and alkalinophilic clusters, but not to the acidophilic one.

Relationships between AOA community compositions and potential nitrification rates

The changes in nitrification enzyme activities were significantly (although weakly) correlated to changes in the genetic structure of the AOA communities (R = 0.14, P = 0.03, Table 6.3). The relationships between the aforementioned groups (acidophilic, acido-alkalinophilic and alkalinophilic) and the nitrification process were also studied. The structures of all three clusters showed small but significant correlations with changes in nitrification (Table 6.3). However, the significance (P values) of the correlations between NEA and the alkalinophilic cluster (R = 0.155, P = 0.002) was higher when compared with acidophilic (R = 0.165, P = 0.023) and acido-alkalinophilic (R = 0.156, P = 0.021) clusters.

Discussion

Influence of soil parameters on AOA abundance and function

The population sizes of the archaeal ammonia oxidizers were similar and within the range observed in other soil systems (Wessén *et al.*, 2011; Hallin *et al.*,

Table 6.3. Correlations between the AOA community structure, abundance, composition, soil chemical parameters (pH, N-NH_4^+, N-NO_3^-, OM %, clay content % and water content %) and NEA (Relate Analysis) obtained with Primer-E (BEST Test).

	NEA* ($\mu gN.h^{-1}.gdw^{-1}$)	pH ($CaCl_2$)	N-NH_4^+ (mg^{-1} kg)	N-NO_3^- (mg^{-1} kg)	OM (%)	Clay (%)	Humidity (%)
AOA abundance							
April 2010	0.843***	0.834**	NS	NS	0.596*	0.797***	0.685**
June 2010	0.789***	NS	NS	-0.859**	NS	0.913**	0.589*
October 2010	0.733***	0.839**	-0.634**	NS	0.569*	0.893**	0.626**
Overall	0.410***	0.716**	NS	-0.478**	NS	0.744**	0.554**
Nitrifying activity (NEA)							
April 2010	NA	0.837**	NS	NS	0.655**	0.935**	0.725**
June 2010	NA	NS	NS	-0.879**	NS	0.913**	0.660**
October 2010	NA	0.851**	NS	NS	0.628**	0.912**	0.723**
Overall	NA	0.823**	NS	-0.315*	0.564**	0.904**	0.667**
AOA community composition (Pyrosequencing)							
Groups of OTUs							
Acidophilic	0.165*	0.347**	NS	NS	0.222*	0.348***	NS
Acido-alkalino	0.156*	0.473***	0.188*	0.336***	0.335***	0.507***	0.329***
Alkalinophilic	0.255**	0.416***	0.250**	0.158*	0.313***	0.437**	0.256**
Overall	0.143*	0.670**	NS	0.237*	0.385**	0.695**	0.528**

Abreviations: ANOSIM, Analysis of Similarities, NEA, Ammonia oxidizing enzyme activity; AOA, ammonia oxidizing archaeal; NA, not analyzed; NS, not significant ; *** $P < 0.001$, ** $P < 0.01$; * $0.01 < P < 0.05$. The relationship between soil physico-chemical parameters and the AOA community composition was obtained with Global Best test. The relationship between soil physico-chemical parameters, NEA and *amo*A gene abundance were obtained by Pearson's correlation in SPSS 18. Correlations between NEA and AOA community composition were obtained by RELATE Analysis, where values are Spearmans' Rho values (sample statistic). The acidophilic, acido-alkalinophilic and alkalinophilic groups of OTUs were obtained from phylogenetic analysis of the 50 AOA OTUs.

2009; Shen *et al*., 2008). Soil-related parameters, such as soil type (Wessén *et al*., 2011), pH (Hallin et al 2009; Nicol *et al*., 2008, Erguder *et al*., 2009), water content (Tourna *et al*., 2008), fertilizer type and nutrient availability (Hallin *et al*., 2009), may affect the population sizes and community structures of the ammonia oxidizers, and, in turn, the nitrification rates in soils (Hansel *et al*., 2008; Schmidt *et al*., 2007). In our study, the *amo*A gene abundances were significantly higher in the soils with higher pH and clay contents (soils G and K) than in soils with lower pH and clay content (B and D) throughout the season. Decreases in AOA abundances have been also observed both with decreasing (He *et al*., 2007; Hallin *et al*., 2009) and increasing (Bates *et al*., 2011; Bru *et al*., 2011) soil pH. Wessén *et al*. (2011) have found that the abundance of AOA was negatively affected by clay content, in an organic farming system. However, we consistently found positive and strong correlations between pH/clay content and AOA abundance and activity. We hypothesize that particular lineages within the AOA might be the main responsible for nitrification in agricultural soils with a long history of N fertilization.

Organic matter and nitrate were also observed as important drivers of AOA abundance and function. The positive correlation suggests that soil OM provides significant carbon to the local AOA communities. Indeed, sequencing of the *Cenarchaeum symbiosum* and *Nitrosopumilus maritimus* genomes suggested that these organisms may be capable of mixotrophy (Hallam *et al*., 2006; Walker *et al*., 2010), as was demonstrated for some Archaea (Crenarchaeota) which can grow heterotrophically (Herndl *et al*., 2005; Ouverney and Fuhrman 2000; Jia and Conrad 2009). Together, these data indicate that AOA might not be solely sustained by ammonia oxidation and that the primary process carried out by these organisms might be methanotrophy or even denitrification. Moreover, the increased abundance with increase in soil OM might be expected, as OM mineralization provides low but constant levels of ammonia (Stopnisek *et al*., 2010).

Nitrogen availability is also known to influence the AOA communities in soil, but the effects are controversial. Nitrogen fertilization has been found to affect the size of the AOA populations (Hallin *et al*., 2009), with, remarkably, much lower values being found in treatments with ammonium sulfate. Some studies have suggested that the rate of archaeal ammonia oxidation is not influenced by substrate concentration (Verhamme *et al*., 2011), whereas in others ammonia seems to promote the growth as well as activity of AOA (Tourna *et al*., 2011). Moreover, it is as yet unknown whether ammonia or the protonated form ammonium is the main substrate for AOA (Martens-Habbena and Stahl, 2011). Our results point towards ammonia as the main substrate, as nitrification rates were much lower in acidic soils. In low pH soils, most of the ammonia may be protonated to ammonium, decreasing its availability for uptake (Valentine, 2007), as previously suggested (Zhang et al 2011). Moreover, previous studies have showed that AOA are more abundant in soils with lower levels of avail-

able nitrogen (Jia and Conrad, 2009), supporting our findings of N (nitrate and ammonium) inhibition of abundance and potential nitrification activity.

Temporal dynamics of the AOA community composition

Seasonal dynamics of microbial community structures have fundamental implications for our understanding of how they function and interact with the environment (Furhman *et al.*, 2006; Yagi *et al.*, 2010). The phylogenetic analysis of the thaumarchaeal *amo*A genes performed by us revealed the existence of highly dynamic communities, with – in each soil - only 25% of the OTUs being shared across time. These results might be due to seasonal changes in temperature, moisture or nutrient status of the soil. In any case, they are remarkably, as they take away our notion of stability, in terms of the structure of a functional community, of the living soil. Szukics *et al.* (2010) studied the effect of increasing temperature (5º to 25ºC) in pristine forest soils and observed a rapid shift in AOA community structures, indicating highly dynamic populations. In our study, such seasonality was also reflected in the diversity of AOA populations, which was higher at the beginning of the season (April and June) than in October. This differentiation of AOA community structure during the fall compared to spring and summer was also observed by us (Pereira e Silva *et al.* 2012) using community fingerprinting (PCR-DGGE) based on the *amo*A gene. Moreover, Wang *et al.* (2012) investigated the seasonal dynamics of AOA in an alpine forest in western China and also observed changes in AOA communities that were mainly attributed to changes in soil temperature and nutrient availability.

These effects might be soil type dependent, influencing ammonia oxidizers in a soil-type specific manner. Soil type has indeed been proposed as a key factor influencing ammonia oxidizers and the resulting nitrification activity (Morimoto *et al.*, 2011), especially the AOA communities (Wessén *et al.*, 2011). Moreover, Pereira e Silva *et al.* (2011) showed that changes in AOA community structure were significantly different between sandy and clayey agricultural soils in the Netherlands. The AOA community composition in the sandy soils B and D did not diverge much, although it fluctuated over time, whereas the AOA community compositions in the clayey soils were different at each sampling time (Fig. S3). These findings go against the proposed theory that unsaturated soils with high clay content provide better conditions for species to coexist (Tiedje *et al.*, 2001), and suggest that other factors, or an interactions of factors, might also play a role in determining AOA diversity in clay soils.

Very few OTUs, between 1 to 3, were shared between all four soils at each sampling time, however there were core sets of OTUs that typically appeared in all time points per soil. This suggested that each soil, at the level of the AOA *amo*A gene, contained a specific unique core AOA community. Furthermore, the higher number of shared OTUs among sandy or clayey soils as compared to the number of shared OTUs among the individual soils suggested that soil type,

possibly reflected in the pH and clay content values, had an important role in determining the AOA community composition. This overriding effect of soil type was potentially stronger than the specific characteristics of each site; it was clear from the NMDS of all OTUs (Fig. 6.3).

Relationships between soil parameters, AOA community composition and function

Results from the phylogenetic analyses revealed three main clusters of OTUs, which correlated with the factor soil pH. The potentially differential responses of AOA OTU clusters to pH is consistent with recent findings that state that some AOA are specialized in high pH environments and others to low pH (Gubry-Rangin *et al.*, 2012). Organisms of cluster I, considered to be well adapted to high pH conditions (more than 80% of the cluster; 11,035 sequences), may have developed mechanisms that allow them to overcome the inhibitory effects of high ammonia concentration. Organisms belonging to cluster II are apparently specific to low-pH environments (99.5% of the cluster; 6,342 sequences). In the study from Gubry-Rangin and coworkers (2012), the acidic clusters were affiliated with groups 1.1a and 1.1a associated, whereas alkalinophilic clusters were affiliated with groups 1.1b.

Interestingly, we observed that the composition of the different clusters correlated with distinct soil factors and did so to different extents. Moreover, their ability to explain changes in potential nitrification rates also varied. N availability was a key factor that determined the overall composition of the AOA communities, and also the composition in acidic-alkaline and alkaline clusters. However, this was not the case in the acidic environments, where OM was more important. This is not surprising, as in acidic soils the levels of ammonium tend to be higher than in alkaline soils, and OM mineralization becomes a key process from which to obtain ammonia. This result also indicates that this group of OTUs might not be just autotrophic but instead, as discussed above, be involved in mixotrophic processes (Walker *et al.*, 2010; Tourna *et al.*, 2011) or even denitrification. This is supported by work by Santoro *et al.* (2011), who suggested that ammonia-oxidizing archaea may be largely responsible for the oceanic N_2O source. Although some other studies have suggested that N availability does not influence the AOA community (Di *et al.*, 2009; Ke and Lu 2012), we observed that in the acidic-alkalinophilic and alkalinophilic groups of OTUs, ammonium (alkalinophilic) and nitrate (acido-alkalinophilic) were significant. This is in coherence with the effects of higher ammonia concentration than ammonium, at high pH. Long-term fertilization practices were found to cause an increase in the abundance of AOA next to pronounced changes in their community composition (He *et al.*, 2007), especially when a combination of NPK fertilizer was used. Long-term application of N fertilizer could also result in soil acidification (McAndrew and Malhi, 1992).

Differences in nitrification rates could be driven by abiotic factors, e.g. pH and N availability (Nugroho *et al.*, 2005, 2007) and soil moisture (Breuer *et al.*, 2002; Corre *et al.*, 2003). However, the most important factor is often the presence of the types of nitrifying microorganisms themselves (Jiang *et al.*, 2011). Correlation between NEA and the acidophilic group were significant. Recently, the discovery of *N. devanaterra*, an obligate acidophilic ammonia oxidizer, which growth is restricted to pH in the range of 4.0 to 5.5Lehtovirta-Morley *et al.*, 2011) provided evidence that the acidic cluster exemplified by this organism may play a major role in ammonia oxidation in acidic soils (Gubry-Rangin *et al.*, 2011). Although slightly negative correlations have been found between NEA and pH (Booth *et al.*, 2005), ammonia oxidation could still occur n acidic soils through growth in biofilms and aggregates (de Boer *et al.*, 1991; Allison and Prosser, 1993)and also in connection to ureolytic activity (Burton and Prosser, 2001; Lu *et al.*, 2012), giving AOA a growth advantage under nutrient-limiting conditions. The release of ammonia molecules, produced upon hydrolysis of urea, can bind protons and consequently elevate the pH (Mols and Abee, 2008). However, it was found this mechanism occur independently of extracellular pH in the range of 4.0 to 7.5 (Burton and Prosser, 2001). Nitrification in acid soils might therefore result from the selection of the acidophilic OTUs which activities may reflect different physiological characteristics.

Moreover, RELATE analysis showed that the significance of correlations between NEA and the alkalinophilic group was higher than between the other groups of sequence, although correlations were weak. The weak correlations between NEA and groups of AOA might suggest that AOA were not the major oxidizers of ammonia in these soils. These suggest a prominent role for AOB, as previously observed by Di *et al.*, (2009) in nitrogen-rich grassland soils. Moreover, the results from our RELATE analyses are coherent with findings of higher AOA abundance and higher activity rates in soils of higher pH (pH above 7.0), and might suggest a bigger role of this group in nitrification, in the agricultural soils analyzed here.

This study suggests that the archaeal clusters commonly found in soils are well adapted to specific pH ranges and they also respond differently to other soil parameters, which has consequences to nitrification rates and important implications for management strategies.

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Table S1. Soil characteristics measured in this study.

Abiotic parameter	Sampling time	Soils			
		Buinen (B)	Drovendaal (D)	Kollumerward (K)	Grebbedijk (G)
pH	April	4.20	5.00	7.40	7.20
	June	4.60	5.10	7.40	7.00
	October	4.40	4.70	7.40	7.40
	Average	4.40	4.93	7.40	7.20
	C.V.	0.05	0.04	0.00	0.03
OM	April	4.90	2.60	6.60	6.40
(%)	June	3.50	3.30	3.30	4.30
	October	3.60	2.60	2.70	5.50
	Average	4.00	2.83	4.20	5.40
	C.V.	0.20	0.14	0.50	0.20
Water content	April	13.80	9.50	16.70	18.80
(%)	June	7.50	16.00	19.70	20.40
	October	12.30	9.40	21.50	19.60
	Average	11.20	11.63	19.30	19.60
	C.V.	0.29	0.33	0.13	0.04
$N\text{-}NO_3^-$	April	24.50	67.90	43.60	59.00
(mg/kg)	June	103.60	68.50	14.80	12.20
	October	13.40	45.20	15.40	18.60
	Average	47.17	60.53	24.60	29.93
	C.V.	1.04	0.22	0.67	0.85
$N\text{-}NH_4^+$	April	10.40	18.40	9.80	19.80
(mg/kg)	June	12.80	15.30	6.60	21.20
	October	4.50	3.20	8.80	4.40
	Average	9.23	12.30	8.40	15.13
	C.V.	0.46	0.65	0.19	0.62

OM = organic matter; $N\text{-}NO_3^-$ = nitrate and $N\text{-}NH_4^+$ = ammonium. Numbers are average of three replicates

Table S2. PCR and cycling conditions for real time PCR and pyrosequencing of archaeal *amo*A genes

	PCR mixture	Thermal conditions
Primers qPCR (5′-3′)		
amo23F (ATGGTCTGGCTWAGACG) (Tourna *et al*., 2008) CrenamoA616r48x GCCATCCABCKRTANGTCCA (Nicol *et al*., 2008)	12.5µl Power Sybr Green PCR Master mix, 0.5ul BSA (20mg/ml), 0.5µM each primer and 2µl DNA template	95°C, 10 min, 1 cycle 94°C for 45 s, 50°C for 45 s, 72°C for 45 s, 39 cycles
Specific primers (5′-3′)		
amo23F (ATGGTCTGGCTWAGACG) (Tourna *et al*., 2008) CrenamoA616r48x GCCATCCABCKRTANGTCCA (Nicol *et al*., 2008)	1x Buffer (Roche), 10mM dNTPs (Roche), BSA 3%, 0.2 mmol/µl each primer, Taq polymerase 1.25U (Faststart High Fidelity, Roche), 50ng template DNA	95°C, 5 min, 1 cycle 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, 5 cycles 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, 25 cycles 72°C 10 min

Table S3. Richness estimates and diversity indices for forward and reverse amplicon libraries at 90% similarity cutoff.

Library	NS[a]	OTUs[b]	Estimated OTU richness		Shannon[c]	ESC[d]
			Chao1	ACE		
Forward						
B_Ap	1963	56	407.5 (174.40; 1099.48)	164.75 (103.88; 303.03)	1.44 (1.37; 1.51)	0.90
D_Ap	695	24	46 (29.58; 110.73)	53.59 (32.75; 124.03)	1.24 (1.13; 1.35)	0.97
K_Ap	1954	27	46.5 (32.21; 99.91)	48.57 (33.72; 96.26)	0.99 (0.94; 1.05)	0.90
G_Ap	1992	25	77.5 (39.27; 218.10)	59.60 (36.06; 133.30)	0.54 (0.49; 0.59)	0.90
B_Ju	1540	34	55.85 (40.98; 102.43)	87.75 (54.02; 178.32)	1.10 (1.03; 1.18)	0.92
D_Ju	1420	33	59.25 (40.44; 125.53)	57.58 (41.38; 105.08)	1.83 (1.78; 1.89)	0.93
K_Ju	2136	30	45 (34.57; 79.15)	56.14 (38.86; 107.13)	0.43 (0.38; 0.49)	0.89
G_Ju	1803	24	31.85 (25.96; 55.46)	35.95 (27.55; 64.18)	0.54 (0.48; 0.59)	0.91
B_Oc	1569	32	117.5 (56.95; 324.96)	91.97 (52.58; 206.70)	1.34 (1.288; 1.41)	0.92
D_Oc	1630	42	88.2 (57.33; 181.14)	82.66 (57.63; 147.83)	1.74 (1.68; 1.80)	0.92
K_Oc	1690	21	32.25 (23.65; 68.72)	34.34 (24.87; 66.89)	0.36 (0.31; 0.42)	0.92
G_Oc	1946	33	96.33 (52.20; 241.86)	96.43 (56.23; 206.21)	1.02 (0.95; 1.08)	0.90
Reverse						
B_Ap	2134	47	78.66 (57.39; 143.45)	77.43 (59.09; 123.55)	1.55 (1.49; 1.62)	0.90
D_Ap	742	24	33 (26.13; 62.02)	39.84 (28.86; 75.62)	1.15 (1.04; 1.25)	0.97
K_Ap	1891	38	62.43 (45.97; 112.84)	71.14 (50.66; 124.75)	0.68 (0.62; 0.76)	0.91
G_Ap	2100	51	93 (67.09; 160.63)	96.86 (70.72; 157.58)	0.89 (0.82; 0.96)	0.90
B_Ju	1603	40	64.43 (47.97; 114.84)	82.77 (56.94; 147.93)	1.24 (1.16; 1.31)	0.93
D_Ju	1589	42	73.66 (52.39; 138.45)	78.16 (56.38; 132.95)	1.87 (1.81; 1.93)	0.93
K_Ju	1956	50	86.14 (62.65; 153.23	85.67 (65.05; 134.61)	1.36 (1.29; 1.43)	0.91
G_Ju	2014	50	86.14 (62.65; 153.23)	85.67 (65.04; 134.61)	1.36 (1.29; 1.43)	0.91
B_Oc	1751	46	76 (57.15; 126.69)	110.89 (74.23; 195.16)	1.43 (1.36; 1.49)	0.92
D_Oc	1641	50	81.63 (61.21; 139.19)	84.88 (64.65; 133.05)	1.95 (1.89; 2.02)	0.92
K_Oc	2132	36	47.66 (39.45; 75.40)	58.35 (44.11; 97.62)	1.18 (1.12; 1.24)	0.90
G_Oc	2140	43	89.2 (58.33; 182.15)	83.56 (58.91; 146.44)	1.22 (1.15; 1.28)	0.90

[a]Number of sequences in each library.
[b]Calculated with DOTUR at the 10% distance level.
[c]Shannon diversity index calculated using DOTUR (10%).
[d]Estimated sample coverage: Cx = 1 - (Nx/n), where Nx is the number of unique sequences and n is the total number of sequences.
Values in brackets are 95% confidence intervals as calculated by DOTUR.

Table S4. Distribution of the most abundant OTUs in the twelve samples analyzed.

OTUs	B			D			G			K			Total
	April	June	Oct	April	June	Oct	April	June	Oct	April	June	Oct	
OTU 1							1		1			9	11
OTU 2	2							2	5		1	1	11
OTU 3						1		6	2	2			11
OTU 4							10		1	1	1		13
OTU 5							5	7	1				13
OTU 6	1			1	1	1	2	1	1			7	15
OTU 7									1	11	4	1	17
OTU 8	13									7			20
OTU 9	7				1		2	3		1	7	5	26
OTU 10	25		1										26
OTU 11	25	1		2									28
OTU 12	27	1			1								29
OTU 13	5	11	6	5	3	3							33
OTU 14	9						1	1	10	3	6	3	33
OTU 16	1							26	8				35
OTU 17	1					1	1	2	21	8	1		35
OTU 19	28	11	8	4	2	1							54
OTU 20		2		55	4	4							65
OTU 21	33	1		1		38							73
OTU 22					43	24	25						92
OTU 23	21							73	2				96
OTU 24	4	16	20	4	10	11	41						106
OTU 25	6	2	44	23	20	12							107
OTU 26							1	107	3				111
OTU 27	4				2	2	4	7	21	71	3	5	119
OTU 28						120							120
OTU 29	4	92	28	1	7	18							150
OTU 30	3		1	1			5	2	155	47	7	4	225
OTU 31	217	5	1		1	4	1						229
OTU 32	1		1		3	3	1	6		2	120	97	234
OTU 33							278	8		1			287
OTU 34	299		1		2	2	6	10	2	6	22	19	369
OTU 35	4	5	2		6	368							385
OTU 36		32	6	3	2	367		1		1			412
OTU 37	2	5	1		280	134		1					423
OTU 38		5	7	6			1	1	1	5	4	449	479
OTU 39		1	1		240	208	121	1			1	1	574
OTU 40	4	7	10		8	109	1		229	162	86	1	617
OTU 41		1		425	432	34							892
OTU 42							1		143	437	349		930
OTU 43		89	457	94	220	63	29	1	5		1	2	961
OTU 44	1		14		2	4	2	10		1	1	1058	1093
OTU 45	10	565	483	15	15	17							1105
OTU 46	1119	1											1120
OTU 47										1135	4		1139
OTU 48	8	613	446	39	56	12							1174
OTU 49	4		3				1280	2	4		2		1295
OTU 50		1	3		1	4	4	15	1290	2	4	1	1325
OTU 51		1	1		1		5	2	5	6	1481	5	1507
OTU 52	1				26	17	138	1474	7	12	2	2	1679
Total	**1890**	**1513**	**1550**	**680**	**1400**	**1599**	**1966**	**1769**	**1918**	**1921**	**2107**	**1670**	**19983**

OTUs 15 and 18 were removed from phylogenetic analysis.

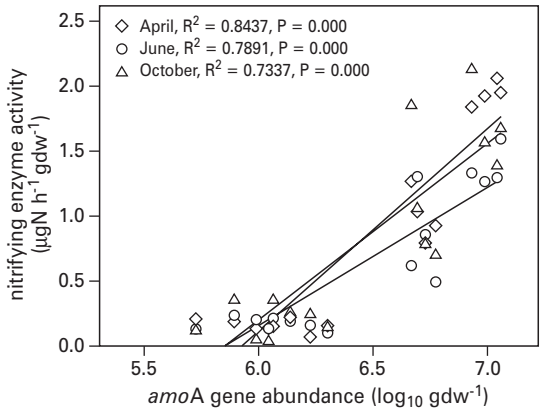

Figure S1. Correlations between nitrifier activity and ammonia-oxidizing archaeal (AOA) abundance, observed in April, June and October of 2010. Each point corresponds to a mean treatment values (n=4).

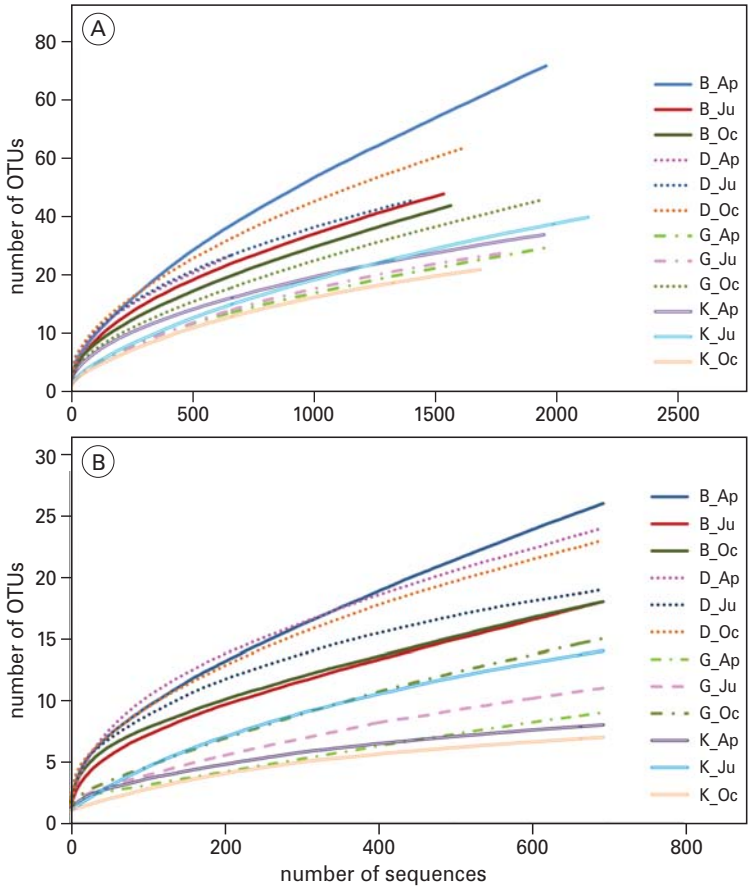

Figure S2. Rarefaction curves of observed operational taxonomic units (OTU) based on total archaeal *amo*A sequences (A) and after resampling to the same sequencing depth (B), determined by DOTUR (Schloss and Handelsman, 2008). B, Buinen; D, Droevendaal; K, Kollumerwaard; and G, Grebbedijk; Ap, April; Ju, June and Oc, October.

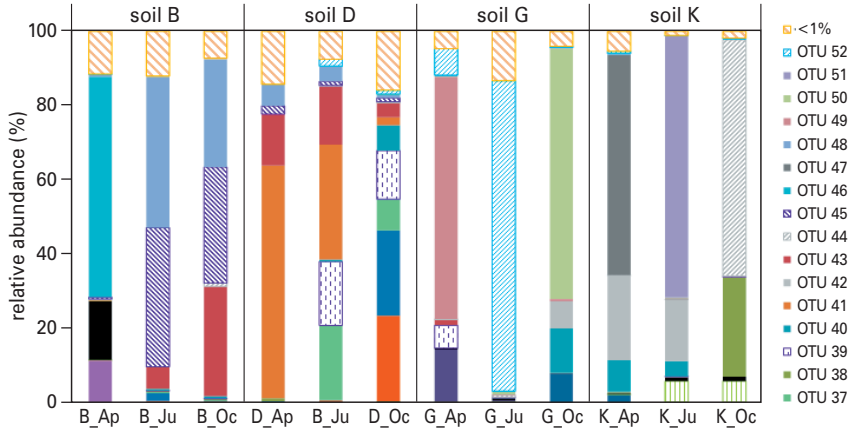

Figure S3. Relative abundance of archaeal amoA (reverse reads) derived OTUs retrieved from four agricultural soils (B, D, G and K) at three sampling times (April, Ap; June, Ju and October, Oc). Sequences were assigned to OTUs using sequence similarity threshold of 90%. The OTUs that had relative abundance below 1% of all sequences were grouped (<1%).

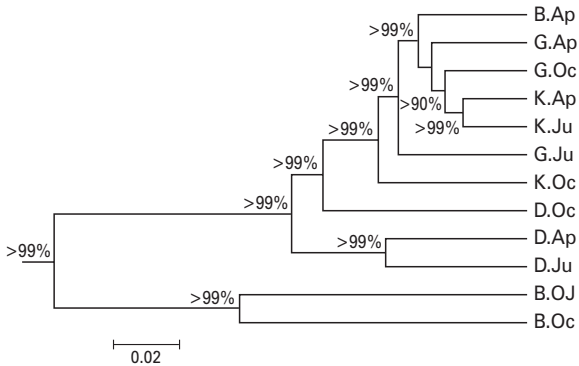

Figure S4. Dendrogram showing the differences in the community structure from the twelve samples. Distance matrices generated with UniFrac were used to cluster the soils using UPGMA; and jackknife analysis was used to evaluate how robust each environment cluster is to sample size and evenness. Numbers indicate the frequency with which nodes were supported by jackknife analysis.

Chapter 7

The influence of soil pH and texture on abundance and functioning of N-cycling microorganisms

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Abstract

The soil microbial communities have been found to respond to several environmental characteristics. Among these characteristics, soil pH and soil type seems to play key roles in how soil microorganisms are structured, and they perform their functions. However, the confounding effects of pH and texture make it difficult to unravel which of them is actually the main determinant of changes in soil microbial community. To unravel these effects we performed a microcosm experiment where a sandy soil with low pH was manipulated. The pH was increased (4.5 to 7.5) as well as the clay content (30% to 80%) by adding montmorillonite, a component of clayey soils. We then, quantified the abundance of key genes of the nitrogen cycle, e.g. archaeal and bacterial *amo*A and *nif*H, responsible for ammonia oxidiation and nitrogen-fixation, respectively. Furthermore we measured potential nitrification activity (NEA). We observed that the addition of montmorillonite had a stronger effect of AOB, whose abundances decreased significantly with increasing clay content. On the other hand, the abundances of AOA and nitrogen-fixers tended to increase with increasing clay content till 60%, after which they decreased. The effect of pH was gene-dependent. Whereas AOB populations increased with increasing pH, AOA abundance decreased and nitrogen fixers were not significantly affected. These results suggest that these different groups might handle different levels of soil disturbances, e.g. liming, with AOA and nitrogen-fixers being more resilient to external changes.

Introduction

The structure and diversity of soil microbial communities have been found to be closely related to soil environmental characteristics (McCaig *et al.*, 2001; Singh and Malhi, 2006), from which soil pH is often strong correlated with changes in composition of soil microbial community (Fierer and Jackson, 2006; Hartman *et al.*, 2008; Jenkins *et al.*, 2009; Lauber *et al.*, 2009). Soil pH is considered at least as important as soil C and N concentrations in influencing the size of the microbial biomass (Wardle, 1992). Soil pH will affect the chemical form, concentration and availability of substrates (Kemmitt *et al.*, 2006) as well as cell growth and activity , and it is known to have a significant effect on the activities of microbial communities and the processes which they mediate (Nicol *et al.*, 2009).

Among other environmental parameters, soil texture also play a role in how microorganisms are structured in soil, their abundance and function (Girvan *et al.*, 2003; Oehl *et al.*, 2010; Chen *et al.*, 2010; Pereira e Silva *et al.*, 2011, 2012). Clay minerals have been shown to be responsible for many sorption and exchange phenomena in soils (Macura and Stotzky 1980). Clay interacts with cells, organic and inorganic nutrients, influencing the physicochemical properties of soil and thus, the activity, ecology and dynamics of soil microorganisms (Stotzky 1972; Filip 1973). The growth of bacteria (Stotzky 1972) and ammonia oxidizing bacteria and archaea (Jiang *et al.*, 2012) were greater in soils containing montmorillonite amendments, a phyllosilicate group of minerals that typically form a clay particle.

Soil texture and soil pH are usually correlated, and sandy soils have usually lower pH compared to clayey soils. This confounding effect between soil pH and texture makes it difficult to unravel which of them is actually the main determinant of changes in soil microbial community. To disentangle the effects of increasing soil pH and clay particles on abundance and activity of soil microbial communities involved in the nitrogen cycling, we manipulated a sandy soil (30% clay content and pH 4.5), by increasing its pH till 7.5 with $Ca(OH)_2$, a practice called liming. Moreover we changed its texture by amending soil with different amounts of Ca-montmorillonite, from 45% till 80% clay content. We quantified changes in archaeal and bacterial ammonia oxidizers, as well as nitrogen-fixers and denitrifiers by real-time PCR amplification during 80 days. Furthermore, we measure potential nitrification and denitrification rates.

Material and Methods

Soil microcosms

The soil (pH 4.5, clay content 30%) used in the microcosm, around 25 kg, was collected in Buinen (The Netherlands). All soil was sieved, thoroughly mixed

and adjusted for the specific pH or clay content. Thirty-five replicate microcosms containing 80 g of soil were constructed in 250 mL bottles (245 in total), fifteen for each different sets of soil pH and clay content, in a destructive sampling scheme. Each pH set contained soil adjusted to pH 5.5, 6.5, and 7.5 with 1, 1.5 and 5 mg g^{-1} soil of $CaCO_3$, respectively. When $CaCO_3$ is added to the soil, it hydrolyzes (dissolves in water) to a strong base, $Ca(OH)_2$, and a weak acid, H_2CO_3. The calcium ions replace absorbed H^+ ions on the soil colloid and thereby neutralize soil acidity. Each clay content set contained soil adjusted to 45, 60 and 80% clay (w/w) with Montmorillonite K10 (Sigma), in triplicates, plus negative controls where only sterile water was added. The properties of montmorillonite were: pH 4.5 and surface area 220-270 m^2 g^{-1}. Afterwards, the soil was adjusted to 55–65% water holding capacity (WHC) which was maintained throughout the experiment by weighing flasks each 2–3 days intervals and drop-wise addition of sterile distilled water to replace that lost through evaporation, and all soil treatments were sterilized by gamma irradiation (Isotron, Ede, Netherlands). After incubation at room temperature (20ºC) for 0, 5, 10, 20, 40 and 80 days, 4 g from each microcosm were separated for immediately DNA extraction from bacterial fraction and another 4g frozen at –20°C. The remaining 70 g of soil were kept at 4ºC and used for determination of pH, $N\text{-}NO_3^-$, $N\text{-}NH_4^+$, moisture and potential nitrification and denitrification activities.

Inoculum preparation

Soil microbial community was extract from Buinen soil for subsequent inoculation. One kg of soil were added to one litter of sterile water, agitated for 1h and left 30 minutes to decant soil particles. In each bottle the same volume of soil suspension was added (3mL) containing 10^7 cells per ml of soils and a remaining 2 to 10 ml was added to each bottle to adjust soil moisture to 65% of water holding capacity. The amount of bacterial cells added was determined by counting them on a Newbauer Chamber.

Soil chemical analysis

The pH was measured in deionized water [using a ratio of 1:2 soil : water (w/v), after shaking for 15 min and settling for 30 min before measurement (Hanna Instruments BV, IJsselstein, The Netherlands). Water content was measured by comparison of fresh and dried (105ºC; 24h) weight of samples. Organic matter (OM) content is calculated as the difference between the initial and final sample weights of dried soil measured after 4 hours at 550ºC. Nitrate ($N\text{-}NO_3^-$) and ammonium ($N\text{-}NH_4^+$) were determined in $CaCl_2$ extracts by a colorimetric method using the commercial kits Nanocolor Nitrat50 (detection limit, 0.3 mg N kg^{-1} dry weight, Macherey-Nagel, Germany) and Ammonium3 (detection limit, 0.04 mg N kg^{-1} dry weight; Macherey-Nagel, Germany) according to Töwe *et al.* (2010) .

Potential soil activities

Nitrifying enzyme activity protocol was adapted from Dassonville *et al.*, 2011. Potential Ammonia–oxidizing enzyme activity (NEA) was determined by using soil suspensions in the presence of non-limiting ammonium and ambient atmospheric O_2 concentration. Plasma flasks (150 mL) containing fresh soil (equivalent to 3 g ovendried soil) and 30 mL of $(NH_4)_2SO_4$ (1.25 mg N. L^{-1}) were incubated on a rotary shaker (180 rpm, 28°C).

The production of nitrate and nitrite during 10 hours of incubation was monitored by periodic (2h, 4h , 6h, 8h and 10h) withdrawal of 2 ml samples by means of a syringe. The samples were filtered at 0.20 µm to remove soil particles and microorganisms. Samples were stored at –18°C prior to analysis. The filtrate was analyzed for nitrate and nitrite content using an ionic chromatography (DX120, Dionex, Salt Lake City, USA) equipped with a 4 x 250 mm column (IonPac AS9 HC). The NEA was calculated from the slope of the linear regression curve of nitrate plus nitrite production versus time.

DNA extraction from bacterial cells

For each sample, the bacterial cell fraction was extracted according to (Duarte *et al.*, 1998). Briefly, 2 g of soil was taken in 15 mL of 0.1% (w/v) NaPP containing 2 g of gravel (2-4 mm diameter), shaken for 10 min (250 rpm), and soil particles and gravel were pelleted by centrifugation (3 min, 121 xg, room temperature). The resulting pellet was re-extracted using 5 mL of sterile 0.1% (w/v) NaPP, spun (3 min, 121 xg) and the supernatant separated. This process was repeated once. The supernatants containing the cells were pooled (total volume 25 mL) and centrifuged (45 min, 8.000 xg), after which the cells pellet was resuspended in 10 mL of sterile 0.1% (w/v) NaPP and subjected to a second high-speed centrifugation step as before. The resulting cell pellet was transferred to the bead tube of Power Soil MoBio kit (Mo Bio Laboratories Inc., NY), where DNA extraction was carried out according to the manufacturer's instructions. The cells were disrupted by bead beating (mini-bead beater; BioSpec Products, United States) three times for 60 s. The quantity of extracted DNA was estimated by comparison to a 1-kb DNA ladder (Promega, Leiden, Netherlands), and quality was determined based on the degree of DNA shearing as well as the amounts of co-extracted compounds. Dilutions for qPCR were quantified in a Nanodrop 2000 (Thermo Fisher Scientific).

Normalization of DNA extraction with Escherichia coli O157:H7 luxA

To normalize the DNA extraction, as DNA can adhere to clay particles, we inoculated *Escherichia coli* O157:H7 derivative strain Tn5 luxCDABE (denoted strain T) in all treatments, re-extract DNA as described above and quantify it using quantitative PCR. This strain is a rifampicin-resistant non-toxigenic derivate of strain O157:H7, which carries a transposon Tn5:luxCDABE construct, allowing

its easy detection from soil. Briefly, strain T was cultured in Luria-Bertani (LB; Sambrook *et al.*, 1989) medium supplemented with rifampicin (10 mg/ml) and kanamycin (50 mg/ml) at 37ºC (with shaking, 100 r.p.m.) for 16h, after which it was used as an inoculant. The soil microcosm with modified texture plus control were inoculated with 1×10^8 cells/ml and left for 4h after which DNA from soil was extracted. A quantitative PCR-based approach (qPCR) was used to quantify *E. coli* strain T in soil environments, using primers Lux-A-fwd (TAC-GCC-AAC-TTG-AAG-ATG-TG) and Lux-A-rev (TCA-TAT-CTG-TGC-CGA-ATA-CG) (Mallon *et al.*, unpublished). qPCR assays were conducted in polypropylene 96-well plates on an ABI Prism 7300 sequence detection system (Applied Biosystems). Each 25-ul reaction contained the following: 12.5 ul of Power Sybr Green PCR Master Mix (Applied Biosystems), 1.25 ul of each primer (10 uM; Eurogentec), 0.5 ul bovine serum albumin (10 mg ml^{-1}; Promega) and 2ul template DNA (~1.0 ng ul^{-1}). PCR conditions were 10 min at 95°C, followed by 40 cycles of 94°C for 1 min, 1 min at 56ºC, and 71°C for 1:30 min. Melting curve analysis of the PCR products was conducted following each assay, to confirm that the fluorescence signal originated from specific PCR products and not from primer-dimers or other artifacts. A plasmid standard containing the target region was generated using DNA extracted from *E. coli* strain T. The amplified products were run on a 1.5% agarose gel to confirm the specificity of the amplification, and products were cloned using the PGEM-T-Easy vector system (Promega). Plasmids were isolated using the Plasmid Miniprep kit (Promega) with DNA concentrations determined using Nanodrop 2000 (Thermo Fisher Scientific). Standard curves were generated using triplicate 10-fold dilutions of plasmid DNA, ranging from 10^6 to 10^2 copy numbers, which were calculated assuming that the average molecular mass of a double-stranded DNA molecule is 660 g mol^{-1}. There was a linear relationship between the log of the plasmid DNA copy number and the calculated threshold cycle value across the specified concentration range ($R^2 > 0.99$; Fig. S1). Amplification efficiency was calculated using the methods described by Pfaffl *et al.* (2001), being in the range of 2.1 across the qPCR assays; these values are consistent with those reported in other studies (Kabir *et al.* 2003; Smits *et al.*, 2004; Stubner *et al.*, 2002). The qPCR assay was further tested and optimized using DNA extracted from eight distinct agricultural soils described previously (Pereira e Silva *et al.*, 2011; Pereira e Silva *et al.*, 2012). In order to test for competitive or inhibitory effects of soil DNA on PCR amplification, a series of mixing experiments were performed combining 2 ul of 10^8 standard plasmid DNA (0.4 ng ul^{-1}) with 2 ul of a ten-fold dilution series of soil DNA (ranging from 10 ng ul^{-1}) to 10 ng ul^{-1}) in 25 ul real-time PCR reactions. We selected samples from two soils, B and G for these tests. Real-time PCR was conducted and the amplification efficiency of mixed DNA was quantified.

Real-time quantitative PCR

The abundance of total bacterial 16S rRNA was quantified with primers 16SFP and 16SRP (both Bach *et al.*, 2002). Crenarchaeal and bacterial ammonia oxidizers were quantified by quantitative PCR (qPCR) targeting the *amo*A gene. For AOA, primers amo23F (Tourna *et al.*, 2008) and crenamo616r (Nicol *et al.*, 2008) were used obtaining fragments of 624 bp. AOB *amo*A quantification was performed using primers amoA-1F (Stephen *et al.*, 1999) and amoA-2R (Rotthauwe *et al.*, 1997), according to Nicol *et al.* (2008), generating fragments of 491 bp. To quantify the number of copies of the *nif*H gene, the primers FPGH19 (Simonet *et al.*, 1991) and PolR (Poly *et al.*, 2001a) were used. The thermal cycling was as described in Taketani *et al.* (2009). The 25µl PCR mixture contained 12.5 µl Power Sybr Green PCR Master Mix (Applied Biosystems, Germany), 0.5 µl bovine serum albumin (Sigma-Aldrich, Germany), 0.25 µM of each primer and 1 µl of template DNA (~1-5 ng/ul). PCR conditions, cycling programs and primer sequences are detailed in Supplementary Table S1. Absolute quantification was carried out twice from each of the four soil replicates on the ABI Prism 7300 Cycler (Applied Biosystems, Germany). The specificity of the amplification products was confirmed by melting-curve analysis, and the expected sizes of the amplified fragments were checked in a 1.5% agarose gel stained with ethidium bromide. Standard curves were obtained using serial dilutions of plasmid containing cloned archaeal or bacterial *amo*A gene, and clone *nif*H gene from 10^6 to 10^2 gene copy numbers/µl. Possible inhibitory effects of co-extracted humid compounds were checked by spiking standard concentrations with samples. No severe inhibition was observed.

Data analysis

Physicochemical variables were checked for normality and were log-transformed, except for soil pH. Correlations between abundance data, activities, soil chemical parameters and NEA were checked by linear regression in SPSS 16.0 (SPSS, Inc, IL), and differences between treatments and over time were assessed with independent sample t-tests.

Results

Changes in soil chemical parameters

From all treatments we measured levels of nitrate and ammonium, pH, humidity, NEA and DEA. The pH of each soil microcosm was checked and adjusted to 4.5 (equivalent to the control) before inoculation. After inoculation, the pH ranged between 4.0 and 4.5 in all clay treatments, being constant until the end of the incubation period. Overall levels of nitrate increased over time in the pH-modified treatments (30% clay content) (from 2.42 ± 0.30 mg/kg dry soil at

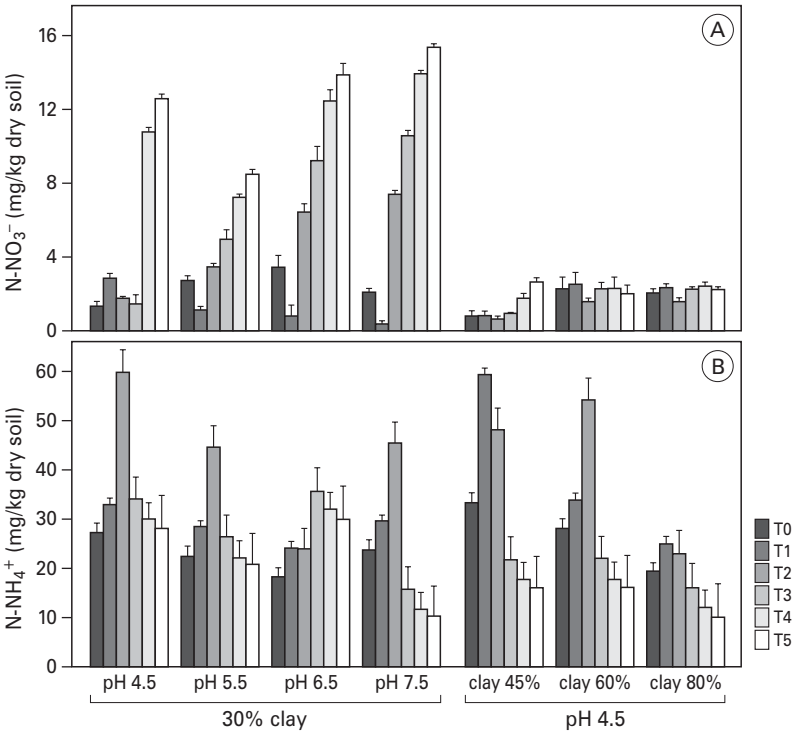

Figure 7.1. Nitrate (A) ($N\text{-}NO_3^-$) and ammonium (B) ($N\text{-}NH_4^+$) quantification in all treatments and times. Bars are standard errors (n = 3).

T0 to 12.58 ± 0.34 mg/kg dry soil at T5) and increased with increasing soil pH (5.14 ± 0.34 mg/kg dry soil at pH 4.5 to 8.28 ± 0.20 mg/kg dry soil at pH 7.5) (Fig. 7.1A). In treatments with modified clay content (pH 4.5), levels of nitrate were much lower and no significant change over time was noticed, except at 45% clay. Levels of ammonium tended to decrease over time in all treatments with a peak at T2, except for pH 6.5, but decreased with increasing soil pH (35.50 ± 12.48 mg/kg dry soil in pH 4.5 to 22.68 ± 22.97 mg/kg dry soil in pH 7.5) and also with increasing clay content (40.51 ± 14.66 mg/kg dry soil at 30% clay to 17.63 ± 4.52 mg/kg dry soil in 80% clay) (Fig. 7.1B).

Overall, NEA increased over time (0.049 ± 0.024 µg.N-(NO_2+NO_3)/h/gdw at T0 to 1.910 ± 0.090 µg.N-(NO_2+NO_3)/h/gdw at T5), and this increase was especially high between T1 and T2 (5 and 10 days), and also between T4 and T5 (40 and 80 days) (Fig. 7.2). NEA also increased with increasing pH (0.611 ± 0.031 µg.N-(NO_2+NO_3)/h/gdw at pH 4.5 to 0.874 ± 0.099 µg.N-(NO_2+NO_3)/h/gdw at pH 7.5). The increase in clay content led to a significant decrease in nitrification rates, which was already noticed in 45% clay treatment (0.0461 ± 0.021

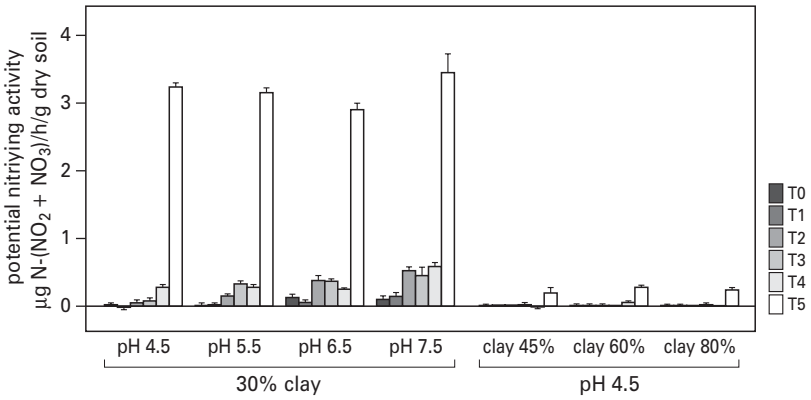

Figure 7.2. Potential nitrifying enzyme activity (NEA) measured in all treatments from T0 (after inoculum) to T5 (after 80 days). Bars are standard errors (n = 3).

µg.N-(NO_2+NO_3)/h/gdw) (Table 7.1), when compared to 30% clay (0.611 ± 0.031 µg.N-(NO_2+NO_3)/h/gdw at pH 4.5). Estimated nitrate levels from nitrification activity were significant correlated with nitrate levels obtained with the Nanocolor kit (R^2 = 0.7839; P < 0.0001; data not shown).

To investigate the possible causes of the shifts observed in both activities, Pearson's correlation was calculated between activity measurements and all soil chemical parameters. We observed that overall changes in NEA were correlated with nitrate (R^2 = +0.915, P < 0.0001), soil pH (R^2 = +0.806, P < 0.0001) and clay content (R^2 = –0.519, P = 0.016). The analyses of the two major treatments (pH and clay content) separately revealed that in the pH treatments, changes in

Table 7.1. Average values of gene abundance and activities observed in the different treatments across time.

	Archaeal *amo*A	Bacterial *amo*A	*nif*H	Bacterial 16S	NEA (µg.N-(NO_2 + NO_3)/h/gds)
pH 4.5 30% clay	4.851 ± 0.122	5.662 ± 0.115	3.542 ± 0.114	8.341 ± 0.073	0.611 ± 0.031
pH 5.5 30% clay	4.674 ± 0.155	5.733 ± 0.131	3.838 ± 0.070	8.255 ± 0.163	0.662 ± 0.034
pH 6.5 30% clay	4.542 ± 0.142	5.894 ± 0.097	3.835 ± 0.126	8.410 ± 0.059	0.684 ± 0.047
pH 7.5 30% clay	4.117 ± 0.056	5.940 ± 0.066	3.750 ± 0.102	8.425 ± 0.094	0.874 ± 0.099
pH 4.5 45% clay	4.950 ± 0.132	5.091 ± 0.074	3.379 ± 0.108	8.016 ± 0.085	0.046 ± 0.021
pH 4.5 60% clay	4.727 ± 0.086	5.363 ± 0.057	3.962 ± 0.067	7.714 ± 0.132	0.068 ± 0.013
pH 4.5 80% clay	4.468 ± 0.086	5.222 ± 0.079	2.020 ± 0.093	6.613 ± 0.147	0.057 ± 0.014

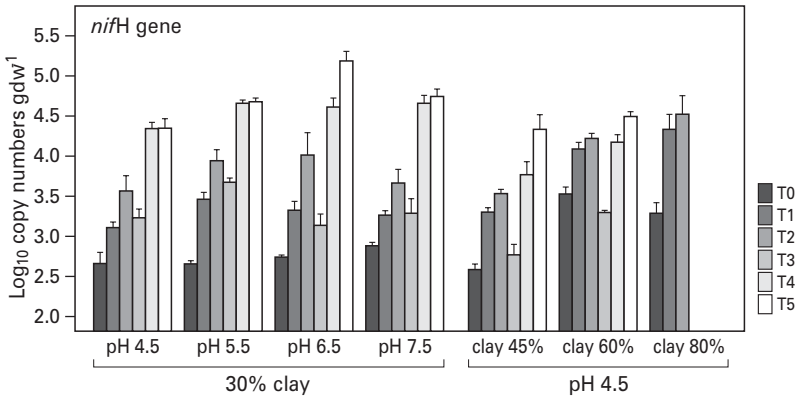

Figure 7.3. Changes in abundance of *nif*H gene in all treatments from T0 (after inoculum) to T5 (after 80 days). The copy numbers per gram of dry soil was estimated by real-time PCR. Bars are standard errors (n = 3).

NEA correlated with nitrate (R^2 = +0.902, P < 0.000) whereas in the clay treatments changes in NEA did not correlated with nitrate nor ammonium levels.

Effect of soil pH and clay content on bacterial *nif*H gene abundance

Overall, the population sizes of nitrogen fixing bacteria, quantified by real-time PCR targeting the *nif*H gene, increased with time in all treatments except in the clay 80%. More specifically, abundances increased from 2.92 ± 0.072 $\log_{10}$ gene copies gdw^{-1} at T0 to 3.96 ± 0.087 $\log_{10}$ gene copies gdw^{-1} at T5 (Fig. 7. 3). All pH treatments revealed the similar patterns through time. In the clay treatments, abundances tended to increase from 30 to 60%. In the treatment with 80% clay, *nif*H gene abundance was under the detection limit already after 10 days. Higher *nif*H gene numbers were observed at pH 6.5 ($\log_{10}$ 3.84 gene copies gdw^{-1} ± 0.125) and at 60% clay content ($\log_{10}$ 3.96 gene copies gdw^{-1} ± 0.06). Comparison between pH and clay treatments revealed that, on average, the treatments with modified pH had higher *nif*H abundances ($\log_{10}$ 3.74 ± 0.103 gene copies gdw^{-1}) than the treatments with increased clay content ($\log_{10}$ 3.12 ± 0.089 gene copies gdw^{-1}) (P < 0.05) (Figure 7.3).

Effect of soil pH and clay content on AOA and AOB abundances, and NEA

Quantification of AOA and AOB were performed targeting the *amo*A gene. Overall, AOA numbers significantly increased from T0 ($\log_{10}$ 3.55 ± 0.128 gene copies gdw^{-1}) to T5 ($\log_{10}$ 6.39 ± 0.052 gene copies gdw^{-1}) (Fig. 7.4A). A significant high increase (almost 100 times) was observed between 20 and 40 days, followed by a smaller increase from 40 to 80 days. This latter increase in AOA abundance with time was higher in pH 5.5 (0.75 $\log_{10}$ gene copies gdw^{-1}) and

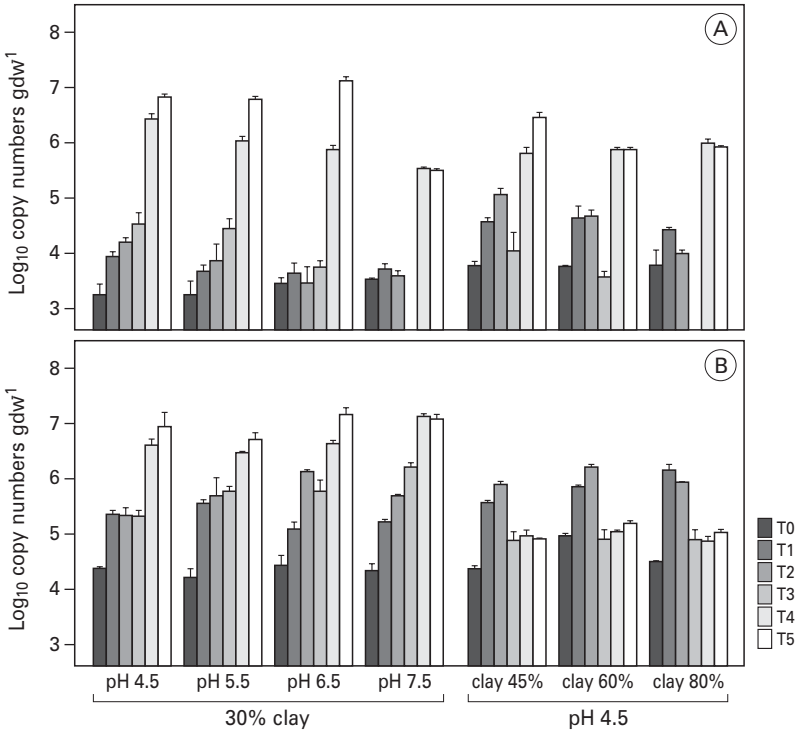

Figure 7.4. Changes in abundance of archaeal (A) and bacterial (B) amoA gene in all treatments from T0 (after inoculum) to T5 (after 80 days). The copy numbers per gram of dry soil was estimated by real-time PCR. Bars are standard errors (n = 3).

6.5 (1.25 $\log_{10}$ gene copies gdw^{-1}) compared to the control (0.41 $\log_{10}$ gene copies gdw^{-1}) but was not significant at pH 7.5. Increasing soil pH had a negative effect on AOA abundance, which decreased from pH 4.5 ($\log_{10}$ 4.85 ± 0.122 gene copies gdw^{-1}) to pH 7.5 ($\log_{10}$ 4.11 ± 0.056 gene copies gdw^{-1}). Increasing clay content led to an overall increase in AOA abundance at the beginning of the experiment (up to T2, 10 days). At T3, a decrease in AOA abundance was observed with increasing content. After this time point, AOA abundances did not differ much from 45 to 80% clay, but were lower that soils containing the lowest clay content (30%). Abundances of AOA were on average higher in the clay treatments ($\log_{10}$ 4.71 ± 0.101 gene copies gdw^{-1}) compared to pH-modified treatments ($\log_{10}$ 4.55 ± 0.119 gene copies gdw^{-1}).

AOB abundance increased over time, from 4.45 ± 0.08 $\log_{10}$ gene copies gdw^{-1} at T0 to 6.14 ± 0.106 $\log_{10}$ gene copies gdw^{-1} at T5) (Figure 7.4B). Separating the samples by pH revealed that AOB numbers were higher at pH 7.5 (5.94 ± 0.067 $\log_{10}$ gene copies gdw^{-1}) compared to pH 4.5 (5.66 ± 0.115 $\log_{10}$ gene

copies gdw^{-1}) when considering all sampling times. AOB abundance decreased significantly with increasing clay content (5.66 ± 0.115 log_{10} gene copies gdw^{-1} in clay 30% to 5.22 ± 0.079 log_{10} gene copies gdw^{-1} in clay 80%) when analyzing all time points. This effect was already detected when comparing 30 and 45 % treatments. AOB abundances fluctuated in a similar manner in treatments with clay contents between 45-80%.

Pearson's product-moment correlations were calculated to test the influence of soil variables on NEA, AOA and AOB abundances. Overall, changes in AOA abundances were driven by ammonium levels (R^2 = +0.597, P = 0.004) whereas changes in AOB abundances were correlated with nitrate levels (R^2 = +0.435, P = 0.049). More specifically, in the pH treatments AOB was significant correlated with nitrate (R^2 = +0.609, P = 0.036) and no significant correlations were observed with AOA. In the clay treatments, significant correlations were observed between AOA and ammonium levels (R^2 = +0.435, P = 0.049), but not between AOB. NEA was significant correlated with both AOA (R^2 = +0.276, P = 0.000) and AOB abundances (R^2 = +0.391, P=0.000) but the correlations were time-dependent (Figure S3). Whereas AOA correlated with NEA at T1 (R^2 = +0.321, P = 0.048) and T2 (R^2 = +0.574, P = 0.049), AOB correlated with NEA at T3 (R^2 = +0.968, P = 0.000), T4 (R^2 = +0.964, P = 0.002) and T5 (R^2 = +0.881, P = 0.000).

Discussion

The influence of soil pH and clay content on AOA and AOB abundance and function

We observed a significant decrease of AOA abundance with increasing soil pH, whereas AOB abundance increased with increasing soil pH. These findings are in accordance with results observed by Nicol *et al.* (2009) in an investigation of whether AOA or AOB were responsible for autotrophic ammonia oxidation. The authors found that both AOA gene and transcript abundance decreased with increasing soil pH (4.9 to 7.5), whereas AOB transcripts decreased with increasing soil pH. Although soil pH is known to drive changes in the AOA and AOB communities, its effects are still controversial. For instance Nicol *et al.* (2008) observed that AOA abundance decreases with increasing soil pH, whereas AOB abundance increases with increasing pH in the soil. On the other hand, He and coworkers (2007) observed significant positive correlations among the population sizes of AOB and AOA and soil pH.

Other factors, such as soil moisture and nitrogen availability, are also known to influence the ammonia oxidizing communities (Hallin *et al.*, 2009; He *et al.*, 2007). In this study soil moisture was kept constant by adding sterile water at a regular basis, and no extra nitrogen was supplied to the soil microcosms. We

observed an increase of nitrate availability over time which also increased with increasing soil pH, suggesting a more active nitrifying population at higher pH, confirming our results based on nitrifying enzyme activity. As expected, an opposite situation was observed for ammonium levels, which decrease with time and also with increasing soil pH. We observed an increase in NEA at the end of the experiment (80 days), and this increase was extreme in the control and treatments with modified pH, reaching values that were up to 7 times higher than those observed for the same soil under field conditions (Pereira e Silva *et al.*, 2012, chapter 4). We could speculate that this rise in nitrification rates was driven by an increase in both AOA and AOB abundances, but also due to heterotrophic bacteria (Balser and Firestone, 2005; de Boer and Kester, 1996), whose abundances increased from T4 to T5 (40 to 80 days; Fig. S2). This proliferation of bacterial cells together with the availability of easily degradable organic substrates that were released during soil sterilization could have lead to higher mineralization rates, providing nitrifiers with NH_4, which was promptly converted to NO_3. Following the same lines, we speculate that this increase in mineralization rates also occurred in the treatments with increasing clay content (pH 4.5), although probably at lower rates than in the pH treatment given the values observed for bacterial abundances (Figure S2). However, due to the adsorption of NH4 to the clay surface, less substrate was available for nitrifiers, leading to an increase in nitrification but to a lower extent than observed for pH treatments.

Clay content affected the two groups of ammonia-oxidizers differently. AOA abundance tended to increase in soils with modified clay content (45%), decreasing afterwards with increasing clay percentage. On the contrary, the addition of clay seemed to be a stronger stress on AOB populations, and numbers were significant lower in clay treatments compared to pH-modified treatments. Although AOB abundance has been found to increase as the rate of montmorillonite amendment increased, from 0% to 12% (Jiang *et al.*, 2012), our results indicate that at higher percentages of montmorillonite (15% to 50%) this effect was reversed. It is known that the attachment of nitrifying microorganisms to soil surfaces stimulates nitrification (Keen and Prosser, 1987). However, we observed that rates of NEA were significant lower in all clay treatments compared to the control. It might be that the lower levels of available ammonium in the treatments which clay was added, as compared to the control, have constrained nitrification.

The influence of soil pH and clay content on nitrogen fixers

Several environmental factors have been suggested to influence nitrogen-fixation in soils, including soil moisture, oxygen, nitrogen availability and pH (Hsu and Buckley, 2009). In the present study, the abundance of *nif*H gene seemed to not be significantly affected by changes in soil pH or in soil texture, although it

tended to increase with increasing pH and clay content until 60%. These results go against previously study where it has been observed higher abundance of nitrogen fixers in high pH soils (7.0-7.5) compared to low pH soils (around 4.5) (Pereira e Silva *et al.*, 2011), and indicates that other parameters excluded in the present study, such as variations in temperature and moisture, commonly encounter under field conditions might also play a role.

Other soil factors such as soil texture and aggregate size (Poly *et al.*, 2001), and clay content (Roper and Smith, 1991), are known to affect nitrogen fixers, as different soil fractions, such as sand-sized and silt-sized ones, have been found to harbor different N fixing populations (Gros *et al.*, 2006). Moreover, clay fractions in soils can form micro- and macroaggregates (Gupta and Roper 2010), and provide microaerophilic or anaerobic conditions that are propitious to nitrogen fixation. In the clay treatments numbers were in general on the same order of magnitude as the pH treatments, except in the 80% clay content, which seemed to be a very strong stress for this community. Indeed, it has been shown that 70% of the free-living nitrogen-fixing bacteria are located in the clay fraction (Chotte *et al.*, 2002). The lack of significant differences between pH treatments and clay contents (except 80% clay), and also the lack of correlation with changes in nitrate and ammonium, might suggest that nitrogen-fixers might represent a nitrogen cycling community that supports better these types of disturbances.

Understanding the effects of soil pH and soil type on microbial abundance and function

Liming is a very common procedure in Dutch agricultural soils. Liming raises the pH and the base cation (Ca and Mg) content of soils, decreasing heavy metal toxicity (Ingerslev, 1997; Kreutzer, 1995). Such changes are probably linked to changes of microbial activities. Indeed several studies have reported higher (Badalucco *et al.*, 1992; Mijangos *et al.*, 2010) and lower (Pawlett *et al.*, 2009) biological activities in limed soils, compared to untreated soils. The influence of soil pH might be an indirect result of a soil-type effect, as clayey soils have higher pH. Here we manipulated both soil pH and clay content in microcosm experiments, in order to assess the specify effect of each one these treatments on microbial abundance and functioning. The experiment was performed for 80 days, after which we assumed that the communities in the soil microcosm were adapted to the new environment, as copy numbers observed for all genes at T5 (after 80 days) were in the same range of values observed for this soil in the field (Pereira e Silva *et al.*, 2011, 2012; chapters 3 and 4).

Overall, we observed a positive effect of soil pH but a negative influence of clay content on all gene abundances and microbial activities. These effects were stronger on bacterial ammonia oxidizers, but archaeal ammonia oxidizers and nitrogen-fixers were affected to a much lesser extent. It might be that the AOB

population inherent to the sandy soil used as control could not survive in a clay environment, which might be too hostile for these communities. Interestingly we observed that the relations of ammonia oxidizers and nitrification rates were dependent on how established the community in the microcosms was (Fig. S3). It might be that nitrification rates were mostly driven by AOA in the beginning of the experiment, due to their ability to growth mixotrophically, but that at the end, when AOB is able to get established, they dominate nitrification rates significantly. If that is the case in general, it might also be that AOA might drive nitrification in soils that have been recently disturbed, whereas AOB might be more relevant in pristine ecosystems. In fact it has been shown that AOA might be adapted to more extreme conditions (Schleper, 2010; Valentine *et al.*, 2007), which does not mean that they are active.

Finally, the results observed in this study suggest that the higher abundance of nitrogen cycling microorganisms observed in clayey soils (Pereira e Silva *et al.*, 2011, 2012, chapters 3 and 4) are more likely a reflect of their higher pH, as previously suggested (Fierer and Jackson, 2006; Hartman *et al.*, 2008; Jenkins *et al.*, 2009; Lauber *et al.*, 2009), and not their texture. These results also might indicate that AOA and nitrogen-fixers are communities more resilient to these types of soil disturbances, compared to AOB.

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Table S1. PCR and cycling conditions for real time quantification of bacterial 16S rRNA, AOA, AOB and nifH.

Primers qPCR (5′-3′)	PCR mixture	Thermal conditions
***amo*A(AOA):**		
amo23F (ATGGTCTGGCTWAGACG) (Tourna *et al.*, 2008) CrenamoA616r48x (GCCATCCABCKRTANGTCCA) (Nicol *et al.*, 2008)	12.5µl Power Sybr Green PCR Master mix, 0.5ul BSA (20mg/ml), 0.5µM each primer and 2ul DNA template	95°C, 10 min, 1 cycle 94°C for 45 s, 50°C for 45 s, 72°C for 45 s, 39 cycles
***amo*A(AOB):**		
amoA-1F (GGGGTTTCTACTGGTGGT) (Stephen *et al.*, 1999) amoA-2R (CCCCTCKGSAAAGCCTTCTTC) (Rotthauwe *et al.*, 1997)	12.5µl Power Sybr Green PCR Master mix, 0.5ul BSA (20mg/ml), 0.3µM each primer and 2ul DNA template	95°C for 10 min, 1 cycle 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, 39 cycle
***nif*H:**		
FPGH19 (TACGGCAARGGTGGNATHG) (Simonet *et al.*, 1991) PolR (ATSGCCATCATYTCRCCGGA) (Poly *et al.*, 2001)	12.5µl Power Sybr Green PCR Master mix, 0.5ul BSA (20mg/ml), 0.25µM each primer and 2ul DNA template	95°C 10 min, 1 cycle 94°C for 60s, 55°C for 27s, 72°C for 60s, 40 cycle
Bacterial 16S rRNA:		
16SFP (GGTAGTCYAYGCMSTAAACG) (Bach *et al.*, 2002) 16SRP (GACARCCATGCASCACCTG) (Bach *et al.*, 2002)	12.5µl Power Sybr Green PCR Master mix, 0.5ul BSA (20mg/ml), 0.8µM each primer and 2ul DNA template	95°C 10 min, 1 cycle 95°C for 27s, 62°C for 1 min, 72°C for 30s, 39 cycle

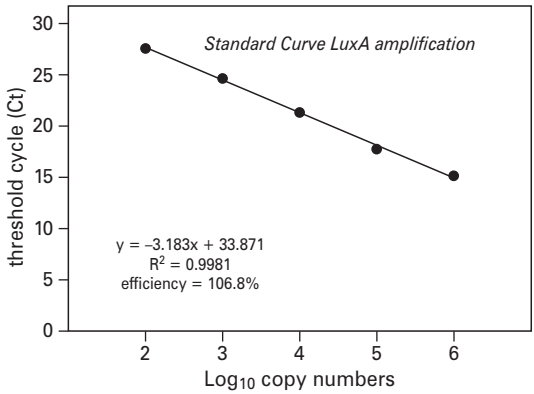

Figure S1. Standard curve obtained from real time amplification of *lux*A gene. Linear regression coefficient ($R^2 = 0.9981$) and efficiency (106.8%) are shown.

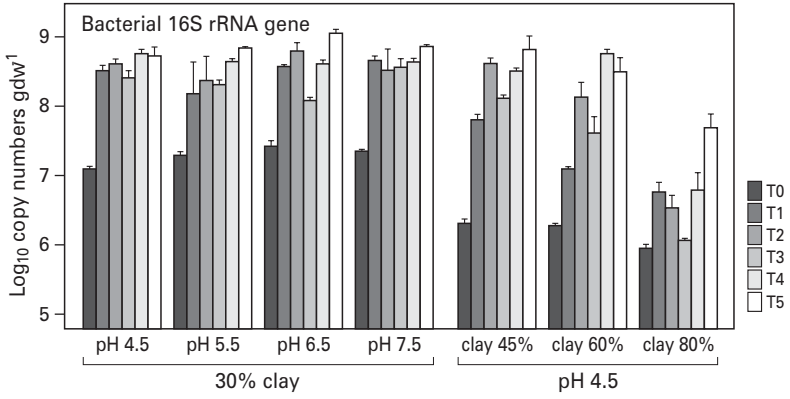

Figure S2. Changes in abundance of total bacterial 16S rRNA gene in all treatments from T0 (after inoculum) to T5 (after 80 days). The copy numbers per gram of dry soil was estimated by real-time PCR. Bars are standard errors (n = 3).

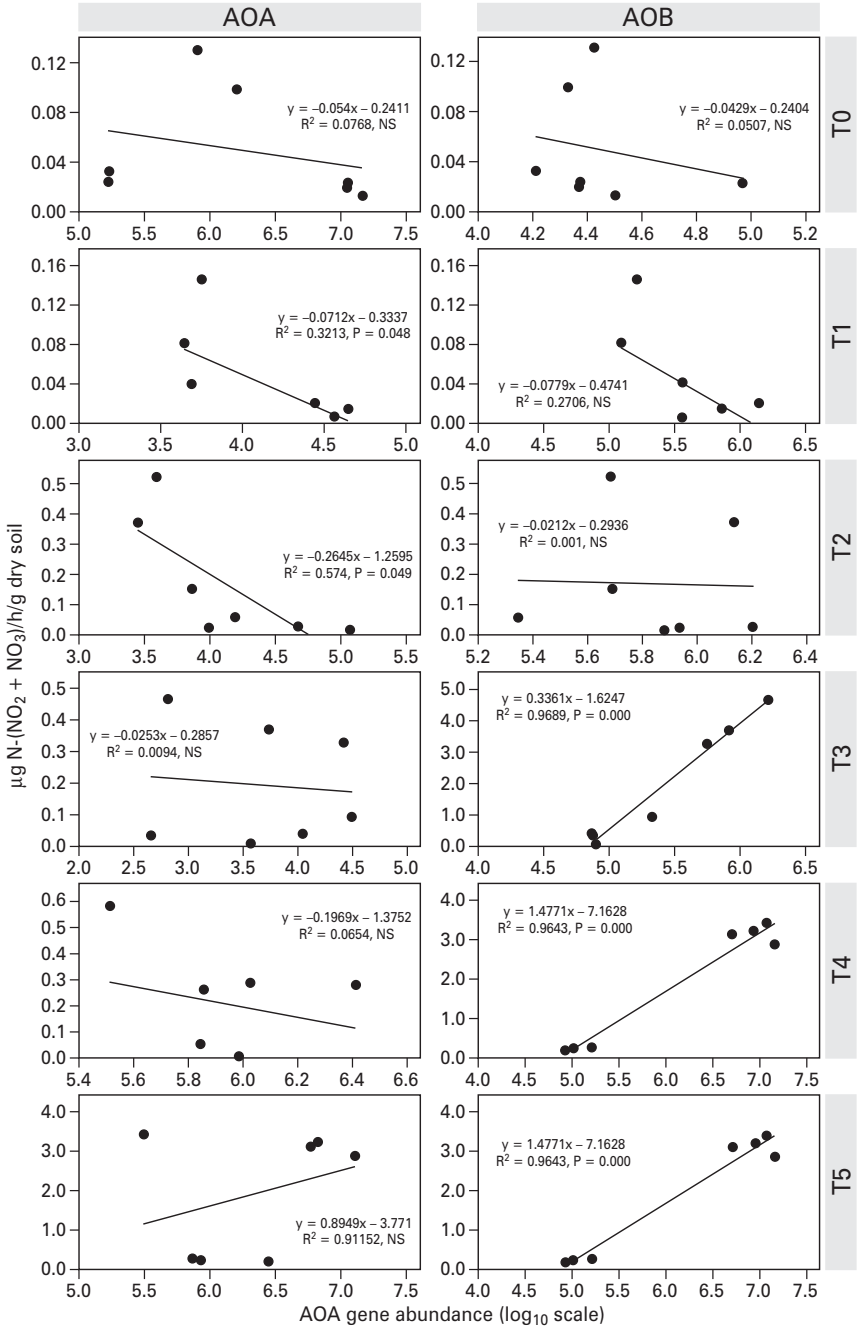

Figure S3. Correlations observed between nitrification activity (NEA) and abundances of AOA and AOB at each sampling time.

Chapter 8

Microbe-mediated processes as indicators to establish the normal operating range of soil functioning

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Abstract

Soils are major contributors to global nutrient cycling processes, which are indispensable for the healthy functioning of our ecosystems. In this study, we raise the question whether soil functioning can be captured in a concept denominated normal operating range (NOR), or the normal fluctuations in soil functioning under field conditions. We further examine how this concept could be effectively used to evaluate the impact of disturbances on agricultural ecosystems. We propose the establishment of a NOR on the basis of multiple parameters in the soil. These should include so-called sensitive processes, that is, those processes that are poorly redundant and easily deviate following a stress situation. The model that we built allowed to visualize the interplay of multiple soil parameters, under which the sensitive ones, which would be most indicative of a disturbance. Here we use the initial step of nitrification, i.e. ammonia oxidation, as an example of a sensitive process. By capturing the normal fluctuations in ammonia oxidation-related parameters that take into account population dynamics, and implementing these in a mathematical model, a multidimensional representation of the NOR of soil function is created which is useful in tests of resilience in the context of disturbances.

The normal operating range (NOR) of soil functioning

Soil is a living entity with global significance. The need to maintain its functions, to sustain biological productivity and to serve as an environmental buffer against disturbances, is large. Soils provide a multitude of ecosystem processes, which have been denoted ecosystem services or life support functions (LSF), in cases of processes that sustain life on Earth. Moreover, soils harbor a great deal of Earth's biodiversity. Microorganisms are key to the living soil, as 80-90% of the relevant soil functions may be mediated by them (Nannipieri *et al.*, 2003). Thus, soil microbial inhabitants play central roles in the maintenance of soil fertility and ecosystem functions, including plant nutrient acquisition (Sprent, 2001; Smith and Read, 1997), nitrogen cycling (Kowalchuk and Stephen, 2001), carbon cycling (Högberg *et al.*, 2001) and soil formation (Rillig and Mummey, 2006). The capacity of soils to sustain such functions is a key feature of what has been denominated "soil health/quality".

It is commonly known that the local environment in which soil organisms dwell is never constant. For instance, conditions such as temperature, water and nutrient availability often fluctuate over time (Schloter, 2003). The extent to which external drivers affect process rates will certainly be dependent on the type of process and associate microbial players. Specifically, such fluctuations may affect the dynamics and activities of soil organisms and the interactions between them and, consequently, the functioning of soil ecosystems (Bascompte, 2009). This natural variation can be depicted as a sequential occurrence of maxima and minima in relevant parameters that define soil process rates (Fig. 8.1). Taken together, such ups and downs determine the 'natural' limits of variation in soil functioning, on the basis of which a normal operating range (NOR) can be defined. Depending on the nature and intensity of the external drivers, higher or lower limits of variation in soil processes may be expected. This description, when used over time, will allow an assessment of the dynamics in the soil status, providing a background against which out-of-range situations are compared (Kowalchuk *et al.*, 2003; Bruinsma *et al.*, 2003). For instance, the characteristics of soil under genetically-modified (GM) plants can be weighed against those in this background, providing a key monitoring tool for policy makers.

Defining soil normality in the context of soil quality

What is expected and can thus be considered to be "normal" in a soil system? This key question is hardly ever addressed in a convincing manner. In line with Rutgers (Rutgers *et al.*, 2009), we propose that normality is defined in relation to the (intended) use of a soil. In other words, the normality range may differ in

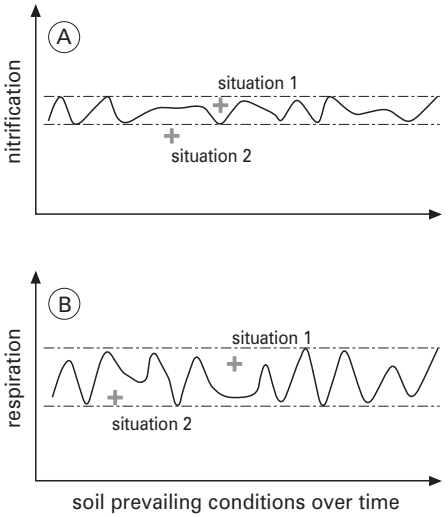

Figure 8.1. Hypothetical behavior of two different soil processes in response to prevailing soil conditions over time, a more specific one, e.g. nitrification (A), versus a more general one, e.g. respiration (B).The fluctuating trajectory represents the normal limits of variation of the mentioned soil process delimiting the normal operating range of soil functioning. In situation 1, the value for both processes falls within the NOR for a given condition. Situation 2 however, fall outside of the NOR only for nitrification (A). For respiration, the intensity of the disturbance has to be much higher to be detected outside of the NOR, as it is a highly redundant process. For nitrification, the limits of variation are lower due to its low redundancy, allowing even subtle changes to be detected.

accordance with whether a soil is used for road construction, for sustaining houses or buildings, or for agricultural or forestry uses. We here will further use the terms NOR and soil normality referring to soils used for plant production, i.e. as providers of the ecosystem services relevant for healthy plant growth and nutrient cycling. Even with this limitation, the values that constitute the "normal" limits of variation of soil processes can differ greatly in dependency of the type of soil, and on its use (agricultural, pristine or grassland) (Bruns et al., 1999; Hallin et al., 2009; Chen et al., 2010; Pereira e Silva et al 2012). Thus, normality will include the expected fluctuations in function in response to all conditions that occur in a particular soil system, either naturally or due to common anthropogenic influences (e.g. its use for agriculture, including soil management practices such as addition of fertilizers). In fact a soil system can fluctuate even in the absence of these external drivers, due to interactions among species (Griffiths and Philippot, 2012). As mentioned above, normality is a soil-dependent feature, as different trait ranges might be expected from e.g. agricultural versus forest soils.

The concept of soil normality should further be linked to observations on soil

health, i.e. an assessment of whether the system is damaged or not. Let us consider soil as a system/machine providing a function, much like our agriculture-based chain provides us with bread or a car engine provides motive force. In such systems, the NOR can be defined as showing a general increase in service output (function) with increasing input. This may be followed by a point at which the output increase declines, as the system falls beyond the "safe range"; structural damage may then be caused. This very point corresponds to the limits of the NOR. If pressure (meaning additional input) is applied after this point, the system will no longer be functioning normally, and will be found to be outside the NOR. These limits are dependent on the resistance of the system to a disturbance and its ability to return to a normal state (resilience) (Seybold *et al.*, 1994; Wertz *et al.*, 2007; Allison *et al.*, 2008). Resistance is intrinsically related to functional redundancy, as more redundant communities may reveal unaltered process rates due to functionally redundant taxa. In fact, many soil communities appear as functionally redundant for a range of functions (Wolters 2001). Resilience is also important, as highly resilient communities will more readily return to their original function following a stress than poorly resilient communities. In conclusion, the concepts of soil resistance and soil resilience are connected to soil stability, which describes the amplitudes of variation in the face of external factors, with more stable systems having lower-amplitude variations (Ives and Carpenter 2007).

Soil quality in the context of the NOR

Given the fact that the soil ecosystem provides valuable key LSF, frameworks for the evaluation and monitoring of the soil status have been designed. Such frameworks are of use in decision-making processes and in environmental policies (Dominati *et al.*, 2010; Robinson and Lebron, 2010) and should reflect, for instance, the sustainability of land management (Herrick, 2000). Soil status includes soil health and/or quality. The latter can be defined as "the capacity of soil to function as a vital living system to sustain biological productivity, promote environmental quality and maintain plant and animal health" (Doran and Zeiss 2000), or as "the capacity of soil to function within ecosystem boundaries and to interact positively with surrounding ecosystems" (Larson and Pierce 1991). At first, assessments of soil quality were intended to serve as tools to assist in the balancing of issues related to increasing worldwide demand for food, feed and fiber as well as environmental protection, and decreasing renewable energy and mineral resources (Pesek 1994, Doran and Parkin *et al.*, 1996, in Karlen *et al.* 2003). In practice, soil quality can be interpreted as a sensitive and dynamic way to describe and scale soil condition and its response to natural or human forces (Arshad and Coen 1992).

The first step in the development of a framework that defines soil quality – and may form the basis of a soil NOR - is thus the selection of an appropriate set of indicators, and their threshold values, at different points in time. Such values would indicate the boundaries of the normal functioning of soil (Arshad and Martin, 2002). Soil quality depends on a large number of inherent and dynamic physical, chemical and biological soil properties, processes and interactions within the soil. Moreover, given the presence of multiple functions in soil ecosystems, such soil quality assessments should include a range of soil attributes which, when considered together, provide an estimation of the biological function of soil (Villamil *et al.*, 2007; Romaniuk *et al.*, 2011; Karlen *et al.*, 2003). Considering that the value that different soil users (stakeholders) attach to different soil LSF may vary, it is important to consider the contribution of a diverse set of users (Rutgers *et al.*, 2012), in the face of the variety of soil and crop management practices being used. The challenging nature of the determination of the most relevant LSF to be included in the NOR for soils might explain why the concept has so far only been applied in a limited number of countries (see Turbé *et al.*, 2010), in spite of the fact that it has been cogitated as from the seventies (Odum, 1979; van Straalen, 2003; Kowalchuk *et al.*, 2003).

Indicators of soil quality

Soil quality indicators measure soil attributes that affect the soil's capacity to support crop production or other LSF (Arshad and Martin 2002). These may include biological, chemical and/or physical measurements. Potential physicochemical indicators of soil quality include pH, cation exchange capacity, organic matter content, bulk density, water retention potential and porosity (Larson and Pierce 1991). Moreover, soil properties such as total carbon, nitrogen, extractable iron and aluminium have been used as indicators of disturbances in forest ecosystems (Silveira *et al.*, 2009). Other examples are soil organic matter, which has been considered as an important indicator of soil quality due to its association with different soil chemical, physical and biological processes (Silveira *et al.*, 2009), and soil biochemical properties, such as microbial biomass, microbial respiration, chitinase and acid phosphatase activities. The latter have been also used as indicators to evaluate the impact of agricultural management regimes (Lagomarsino *et al.*, 2009). Last but not least, soil fauna has been suggested as potential indicators as well (Wolters 2001; Osler and Sommerkorn, 2007), yielding soil quality indices based on microarthropods (Paolo *et al.*, 2010; Yan *et al.*, 2012; Cluzeau *et al.*, 2012).

In spite of the potential utility of the abovementioned indicators, we here advocate a central place for microbial-based indicators in descriptors of a soil NOR, and the rationale behind is threefold. First, the majority of soil LSF is

driven by the soil microbiota (Ritz *et al.,* 2009). Microorganisms indeed make up the largest part of the total biomass in the soil (Brookes *et al.,* 1982; Winding *et al.,* 2005) and are key drivers in processes that contribute to the provision of essential ecosystem services, such as respiration, decomposition of organic matter and nitrification and other N-related processes (Barios 2007). Given the central role of microbes in ecosystem processes, microbial facets of soil are at least as important as the soil's physical or chemical parameters. Even though the relationship between soil microbial diversity and functioning has not been fully unraveled (Hooper *et al.,* 2005), these two facets should be regarded as intrinsically associated (Turbé *et al.,* 2010). Second, microorganisms rapidly respond to environmental stresses, as they have intimate relations with their surroundings due to their high surface-to-volume ratio (Winding *et al.,* 2005). The term microbial community adaptation has been recently coined as "the process by which the observed level of particular trait within a community becomes suited to current environmental conditions" (Wallenstein and Hall 2012). It relates to how fluctuations in specific microbial populations in response to changes in environmental conditions, affect the aggregate function of the community they belong to. Lastly, they can also be easily traced by molecular methods, translating changes in their abundance and diversity into tangible parameters. Thus, a plausible approach to establishing a soil's NOR is to derive parameters from sensitive microbe-mediated soil processes that relate to process dynamics. Moreover, future soil monitoring studies are needed to compare the sensitivity of processes mediated by microorganisms to soil stress in comparison with e.g. community structure of higher organisms.

Identifying key microbial parameters for the NOR

The applicability of the NOR as a monitoring tool relies strongly on its ability to define what is normal in a soil, and thus to detect changes in response to disturbances. It is important to have a broad vision of the key processes that define soil function. Given the fact that most soils can be characterized as being multifunctional, this implies a multi-focused view on the system. Although increasing the number of parameters measured in principle enhances the possibility to detect a change from normality, it also reduces the ease of use. In order to circumvent this trade-off between feasibility, sensitivity and importance, it has been proposed that the selection of key processes for the NOR is to be based on preselected criteria (Schloter 2003; Bruinsma *et al.,* 2003). Briefly, the process parameter should (1) be relevant to the ecosystem under study, (2) reveal a fair response to particular stress factors (stressors) that would put the system outside of the NOR, and (3) be easy to measure, working equally well and reliably in all environments.

In principle, soil activities such as the decomposition and mineralization of a range of polymeric substances, next to steps in the nitrogen and sulfur cycles could be used as process parameters due to their relevance for soil functioning (Mulder *et al.,* 2003; Franchini *et al.,* 2007) (Table 8.1). For instance, the metabolic quotient (qCO_2; respiration to microbial biomass ratio) has been used to indicate transformation ability of the soil microbiota (Anderson, 1994; Turco *et al.,* 1994; Sparling, 1997). The rationale was that any impact on the soil microbiota, such as those caused by changes in temperature, moisture or nutrient status, is reflected in a change of the qCO_2 (Anderson, 1994; Mulder *et al.,* 2005). This parameter appears to fulfill the first two criteria mentioned above, but the underlying function is often highly redundant in soil. Hence, effects of particular impacts that put the system outside of the NOR might not be easily detected (Fig. 8.1). In this context, we argue here that the NOR should be founded in LSF performed by particular non-redundant functional groups, e.g. in ammonia oxidizers (Kowalchuk and Stephen, 2001; Mendum, 2002), methane oxidizers (Hanson and Hanson, 1996), nitrogen fixers (Pankhurst *et al.,* 1995) and/or sulphur mineralizers (Deng and Tabatabai, 1997; Schmalenberger *et al.,* 2008) and/or pollutant degraders (Souza *et al.* 1998; Gentry *et al.* 2004) (Table 8.1). Due to their low redundancy, changes in different components of microbial communities will be more promptly translated into changes in related processes. Thus, their ability to detect subtle changes in the ecosystem make them potential candidates for providing an early warning of soil degradation, avoiding costs of preventing reductions in land productivity (Barrios 2007). From the non-redundant functions mentioned above, nitrogen fixation and nitrification have been advocated as providing quite suitable proxies of soil "normality", in particular in the context of the assessment of the risks of GM plants (Domsch *et al.,* 1983; Kowalchuk *et al.,* 2003; Bruinsma *et al.,* 2003). It is important to note that redundant functions could, in principle, still be included in a model describing the soil's NOR, as long as other lowly- or non-redundant relevant processes are also included in the model (see below).

Nitrification-related parameters as proxies to be included in the NOR of soil functioning

Nitrification is a two-step process in which ammonia is initially converted into nitrite (ammonia oxidation, first and rate–limiting step), which is in turn converted into nitrate (nitrite oxidation, second step). The consequences of nitrification include the contamination of surface and groundwater with nitrate, loss of soil fertility, greenhouse gas emissions and the degradation of agricultural soil (Kowalchuck and Stephen 2001, Gosh and Dhyani 2005; Santoro *et al.,* 2011). The application of nitrogen fertilizer based on ammonia-nitrate clearly

influences the N budget of soils and the corresponding functional microbial groups (Hallin *et al.,* 2009), increasing rates of nitrification and denitrification shortly after application (Le Roux *et al.,* 2003; Patra *et al.,* 2006). Because of its economical and agricultural importance, we placed a focus on nitrification as a key LSF, deriving proxies that support a NOR for agricultural soils. Additionally, the first step of nitrification, ammonia oxidation, may be severely

Table 8.1. Key processes carried out by the soil microbial community.

Key soil processes	Remarks
Respiration and mineralization	These are key soil processes carried out by the biggest part of soil microbial community (Mulder *et al.*, 2003; Franchini *et al.*, 2007). It is highly influenced by changes in temperature, soil moisture and nutrients.
Nitrogen fixation	Symbiotic nitrogen-fixing bacteria are critical to the nitrogen dynamics of many agricultural systems, forming a cohesive group. They are influenced by several soil chemical parameters, e.g. soil moisture, oxygen, pH, carbon quantity and quality, nitrogen availability (Hsu and Buckley, 2009), soil texture (Poly *et al.*, 2001; Pereira e Silva *et al.*, 2011) and clay content (Roper and Smith *et al.*, 1991).
Nitrification	The oxidation of ammonia is the first and rate-limiting step in the nitrification process, crucial for the nitrogen balance of plant-soil systems. Ammonia-oxidizers show a narrow phylogenetic range (Kowalchuk and Stephen, 2001; Mendum, 2002), and some soil properties and environmental conditions can severely affect this community (Hallin *et al.*, 2009; Nugroho *et al.*, 2006; Schmidt *et al.*, 2007; Hansel *et al.*, 2008).
Denitrification	This process contributes to the emission of N_2O, which is an important greenhouse gas with a global warming potential (Demanèche et al., 2009). Members of this functional guild belong to more than 60 genera of bacteria and to some archaea and eukaryotes (Philippot et al., 2007). It is an anaerobic process, very dependent on abiotic factors (precipitation, soil compactation) being affected by management practices.
Methane oxidation	Important role in the global carbon cycle and are potentially useful in curtailing the contribution of methane emissions to global warming (Pankhurst et al., 1995). Methanotrophs comprise 13 genera within the α and γ Proteo bacteria (Dumont and Murrell, 2005). The application of fertilizers can restrict methane oxidation in agricultural areas (King and Schnell, 1994).
Sulfur mineralization	Sulfur is an essential element for plant growth and plants can thus be strongly affected by S deficiencies (King and Schnell, 1994). Arylsulfatases, which catalyze sulfur mineralization, have been studied so far in a variety of bacterial species, and can be affected by soil moisture and temperature, and also by crop rotation and plant cover (Deng and Tabatabai, 1997).
Degradation of recalcitrant organic matter	Recalcitrant organic matter, e.g. lignin and wood can be degraded by some fungi. The process is known to be sensitive to disturbances with low redundancy (Boddy and Watkinson, 1995)

impacted by major impacts on the soil system (Kowalchuk and Stephen, 2001). Thus, this process may represent a suitable set of parameters that are sensitive to disturbances, supporting their use in the soil NOR.

Ammonia oxidation is carried out by particular groups of bacteria and archaea. Ammonia oxidizing bacteria (AOB) have already been proposed as indicators of soil disturbance (Stephen *et al.,* 1999; Oved *et al.,* 2001; Nyberg *et al.,* 2006). The narrow phylogenetic range of these bacteria, their functional cohesiveness and avid response to environmental stresses have made them ready candidates for use in soil health studies (Kowalchuk and Stephen, 2001; Mendum, 2002). In fact, in a recent study comparing 183 different candidate parameters for the assessment of changes in soil properties, genetic profiling of AOB communities was top-ranked (Ritz *et al.,* 2009). Moreover, an analysis of microbial communities involved in N-cycling in over 107 soil sites in France showed that changes in land use did not strongly influence the abundance of any of the studied communities other than the AOB. This indicated that AOB abundance could be used as a pertinent biological indicator for soil monitoring (Bru *et al.,* 2010).

Ammonia-oxidizing archaea (AOA) also play a role in the nitrification (Leininger *et al.,* 2006; Treusch *et al.* 2005), and like their bacterial counterparts, have been also proposed as a microbial group sensitive to disturbances (Wessen and Hallin 2011; Pereira e Silva *et al.,* 2012). In fact, due to their presumed niche differentiation and different susceptibility to environmental change, both AOB and AOA have recently been proposed as potential bioindicators of soil health (Wessén and Hallin, 2011). In this context, both the quantities (abundances) and diversities of the AOA and AOB communities could be included in the NOR.

Proposal of a trait-based space approach to establish the NOR of soil function

The NOR concept has been used in several different areas, such as geochemical science (Wang *et al.,* 2010) and molecular ecology (de Boer *et al.,* 2011). Recently, it has been proposed in microbial ecology (Inceoglu *et al.,* 2011; Rutgers *et al.,* 2009; Pereira e Silva *et al.* 2011, 2012). However, up to now, no appropriate method has been developed that satisfactorily defines the NOR of soils. One proposal currently in use in the Netherlands rather use a specified reference state for comparison, but do not take into account the variability in that reference location (Rutgers *et al.,* 2009). Other methods that integrate several parameters into an overall index neither look at variability (van Wijnen *et al.,* 2012). An interesting approach has been launched by Kersting (1984) in the assessment of the effect of pesticides on aquatic microcosms, in this case by analyzing several variables simultaneously. A similar approach has been suggested (van

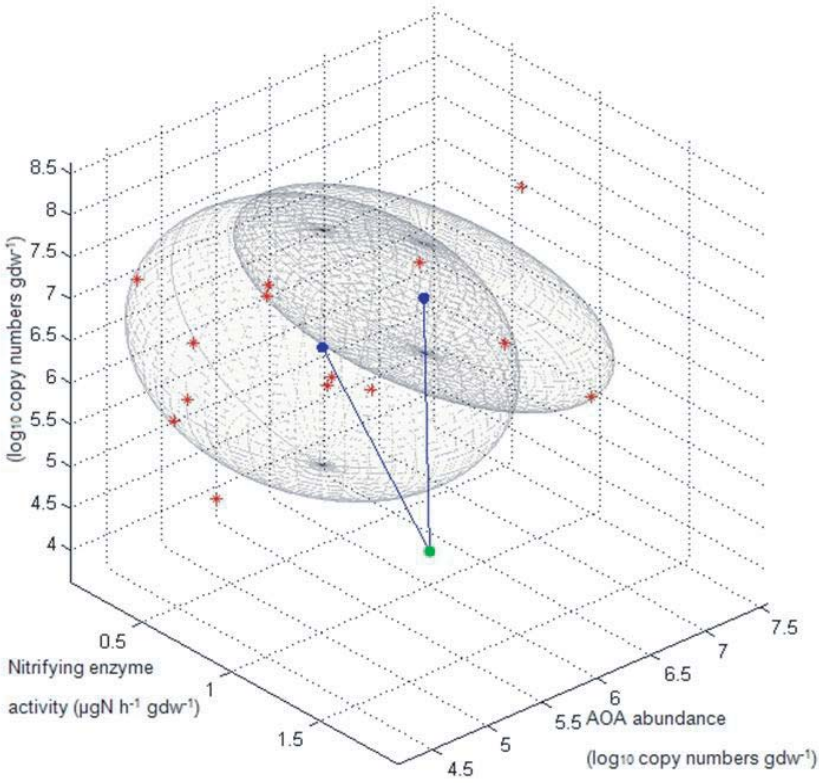

Figure 8.2. Representative example of a NOR of soils showing three of the 22 dimensions. The ellipsoid 1 characterizes the NOR for agricultural soil under tillage while ungrazed grassland is represented by the ellipsoid 2. The ellipsoids represent the borders of the NOR for 3 indicators (nitrifying enzyme activity and abundance of AOA and AOB). Red crosses are observed values which characterize the NOR. The blue line is the distance between the center of the NOR (blue dot) and the state of the selected soil (green dot). It is important to mention that the distance that reflects how much the selected soil (green dot) is outside the NOR is the distance between the green dot and the border of the ellipsoid. Two ellipsoids are different in volume due to higher limits of variation observed in the abovementioned indicators for more disturbed soils (agricultural) compared to the grassland one.

Straalen, 2002) for the detection of ecotoxicological effects of soil pollutants.

Mathematically, the trait-based approach can include numerous parameters. It can thus be depicted as an ellipsoid in a space of n dimensions, where n is the number of parameters measured in a single system, its borders representing the NOR (Fig. 8.2). The distance between a particular state of the soil and the center of the NOR will represent a quantitative measure that summarizes the state of the soil. This was defined by Kersting (1984) as the "normalized ecosystem

strain" (NES). Moreover, the strength of the "stress", or how much a soil is outside the NOR, can be determined by the distance between the "stressed" soil and the border of the ellipsoid. When the soil is in an undisturbed state, all combinations of the parameters fall within the NOR, giving a NES value that is smaller than one unit. Values exceeding 1 would indicate that the system is under "stress". The advantage of this approach relies on the fact that the specification of critical values for each of the selected indicators is not necessary, but only a critical limit considering the whole dataset of indicators, which is 95% confidence are of undisturbed states. The decision, however, whether a deviation of a soil from the NOR is "adverse" or not should be made by an educated guess with respect to the level of potential harm to the system. Ultimately, this would be a decision to be left to decision makers (Smit *et al.,* 2012). The qualification of harm will depend on the use of the soil, e.g. for cultivation in agriculture or for nature development, and can only be done on the basis of the functions of that specific soil under evaluation (Rutgers *et al.,* 2009).

Recently, a study on particular soil parameters across Dutch soils was performed, over two consecutive years (Semenov *et al.,* in preparation). The study aimed to distinguish key soil parameters that could play an important role in the proper establishment of a NOR for soil function. In total, 21 measurable parameters were selected to define the NOR, including soil pH, organic matter, level of nitrate, abundance of bacteria, archaea, fungi, ammonia oxidizers, nitrogen fixers and denitrifiers. Moreover, nitrification and denitrification potentials were measured. We used the model defined by Semenov *et al.* to determine how a NOR based on nitrification-related parameters (activities, abundance and diversities) would performed when compared to model based on full set of general parameters. We then compared the NES values observed for "stressed" soil, which gives an indication of the confidence of the model in detecting a deviation from normality. The results are presented in table 8.2 and show that the distance observed between a "stressed" soil to the NOR part based on nitrification-related parameters (activities, abundance and diversities) was much higher than the corresponding value between the NOR part based on other relevant parameters (e.g. soil pH, OM, archaeal and fungal abundances and diversities) (Table 8.2). The NES value was also higher when compared to the NOR based on more redundant proxies (e.g. denitrification potential and abundance of total bacteria and denitrifiers). These results supported our hypothesis that by focusing on sensitive parameters, such as those describing nitrification (taking the abundance, structure and function of ammonia oxidizers as parameters) a sound NOR of soil functioning can be reliably achieved. Consequently, the chances of distinguishing disturbed soils (measurements outside the NOR) are expected to be higher when a focus is placed on the so-called sensitive parameters than when randomly selected parameters are tested (Fig. 8.2). Based on the aforementioned principles to select indicators and these results, a classification

of potential biological parameters is illustrated in Fig. 8.3, where we top ranked nitrification-related measurements in relation to other more redundant measurements. However, it is worthwhile to mention that the sensitivity of the above mentioned parameters is depend on soil texture, being more sensitive in sandy soils. In fact, previous results indicate that the structure of AOB has been found

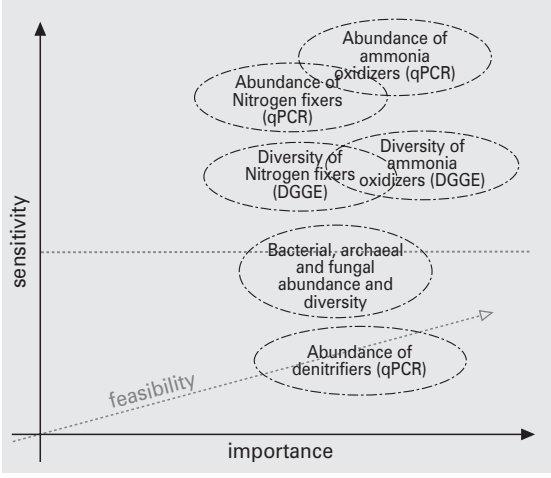

Figure 8.3. Classification of potential biological parameters of soil healthy and the tradeoff between their importance and sensitivity, based on the contention that such parameters should represent ecologically relevant functions, be sensitive to disturbances but also easily measured.

Table 8.2. NES values (distance from the NOR) obtained when comparing a "stressed" soil to NORs based on different indicators. NES values higher than one indicate that a given soil is outside the its normal operating range. Higher values indicate higher confidence in detecting a deviation from normality.

	NOR based on [1]		
Distance from the NOR	All indicators (22)	Nitrification-related indicator (6)	Redundant indicators (5)
"Stressed" soil	32.53	90.77	4.03

[1]All indicators: abundance and Shannon diversity index of total Bacteria, Archaea, Fungi, nitrogen-fixers, archaeal ammonia oxidizers, bacterial ammonia oxidizers, denitrifiers (based on nosZ gene), nitrogen-fixers (based on nifH gene), pH, organic matter, nitrate, ammonium, clay content, potential nitrification (NEA) and denitrification (DEA) activities; Nitrification related indicators: abundance and Shannon diversity index of archaeal and bacterial ammonia oxidizers, and potential nitrification rates (NEA)…; Redundant indicators total bacteria, archaeal and fungi, denitrifiers (based on nosZ gene) and potential denitrification activity. Numbers in between brackets indicate the number of parameters used to define NOR.

to fluctuate more than the structure of AOA communities in sandy soils and that clay content was the main soil factor shaping the structure of both the AOA and AOB communities (Pereira e Silva *et al.,* 2012). These results support the idea that NORs should be built taking into consideration the type of soil under evaluation and provide evidence that establishing one NOR for the functioning of all soils is likely an unrealistic goal.

Despite the utility of the proposed models, it should be clear, however, that they are informative rather predictive. Thus they should be used as a tool that allows users to detect changes in the soil, which might indicate impacts that go beyond the normal soil functioning. Notwithstanding the subtle differences introduced by differential land use and management, in particular variations in soil texture (soil type) should be carefully explored. Thus, NORs for soil function will possibly need to be defined for (1) sandy and (2) clayey soils, and maybe for other soil types (e.g. silty, high-organic). It is plausible that land use and/or management practices are included into these major NOR types. For each soil type, the model that mathematically describes the NOR may subsequently be trained, enabling a comparison of the data obtained on presumably healthy versus disturbed soils under various land use and management practices. This will fine-tune the definition of the NOR, as well as the level (management/land use) at which it applies better, allowing the detection of systems under potential stress as a deviation from its 'normal' condition.

Prospects

As argued in this paper, the soil microbiota, that underlies the key LSF processes of soil, is utterly complex. We are still far from being able to exhaustively sample and analyze all aspects of the microbiota of a soil, and hence depend, for our assessments of soil healthy and normality in soil functioning, on proxies that define our capacities of analysis. Moreover, our understanding of the putative link between soil microbial diversity and community make-up and soil functioning (the holy grail of soil microbial ecology), is fragmentary, to say the least. This inevitably leads to the conclusion that, on the basis of current technologies, it is impossible to come to grips with the full complexity of the mechanisms and interactions that impact on the key processes of soil.

To address the NOR of soil functioning, one should thus resort to measurable key processes, including disturbance-sensitive ones, in a soil, as these might provide indications that the soil system is disturbed, i.e. outside the normal operating range. We here suggested that the first step in nitrification, i.e. ammonia oxidation, represents one example of a well-measurable disturbance –sensitive process. However, to use the parameters defining this process as proxies in the overall soil NOR, this requires us to define and characterize what is regard-

ed as a reference (standard) soil condition, taking into account the natural dynamics of the processes and communities. We thus propose that, through long-term analyses of abundance (qPCR) and diversity (e.g. using PCR-DGGE) of the ammonia oxidizers per soil, defining the NOR, as well as deviations from it, will be conceivable. One should take into account that soil type is a key facet in this endeavor (Pereira e Silva *et al.,* 2011, 2012). In fact, the relevance of soil type when defining the NOR has been also mentioned in studies focusing on soil macroorganisms. For instance, gene expression in the soil-dwelling collembolan *Folsomia candida* was differentially regulated in a clayey versus a sandy soil (de Boer *et al.,* 2011). Moreover, soil management regimes and land uses should also be considered, possibly leading to a fine-tuning of the NOR per soil type.

As argued, the NOR of a soil can be mathematically described in a model that is based on multiple parameters, in which those describing a sensitive process are primordial. In order to come to such a depiction, we have taken a model developed to describe fluctuations in population dynamics and applied it to soil processes, giving nitrification a central place. A multidimensional depiction of the NOR space, compared to simulated deviations from it, allowed a clear visualization of stressed situations. Thus, a prototype-monitoring tool for educated judgment of soil normality was created. Moreover, we can estimate the intensity of the stress applied by measuring the distance between the center of NOR and the actual state of a selected soil, with bigger distances reflecting larger disturbances. However, the real impact of the stress on soil functioning and the amount of time required for the soil to recover from the stress and return to within its NOR (resilience) cannot be inferred. For that, information present in larger datasets should be used for establishment of the soil NORs. In this, the choice of the parameters should be considered, as well as the distance to the NOR space. Other parameters related to stress also need consideration to feed models that will allow us to infer soil functioning.

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Chapter 9

Quantitative assessment of soil functioning across a representative range of Dutch soils

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Abstract

Soil microorganisms are the most important determinants in the soil functioning. In order to understand the relevance of stressor-induced changes, the natural variation (or normal operating range, NOR) of soil function caused by complex stress effects (e.g. GM plant) has to be better understood. Quantitative assessment of the NOR taking into account the most important and most sensitive microbial groups may lead to the first quantitative characterization of the baseline of an entire soil system. The focus is on the quantitative measurements by real-time PCR as well as diversities by PCR-DGGE of the key genes involved in the nitrogen cycle (*amo*A and *nif*H), next to other soil and microbial measurements. The NOR can be considered as a space of n dimensions, where n is the number of variables measured. When the soil is not disturbed, all combinations of the variables fall in the NOR. The distance between an investigated state and the center of the NOR represents a quantitative measurement that summarizes the state of the soil, taking into account the multivariate nature of the data. The parameterization of the model was done by performing several microcosm experiments as well as by sampling selected soils in natural conditions during subsequent 3 years. 21 parameters were measured for the calculations, resulting in a space with 21 dimensions. One of the advantages of the approach is that the data itself shows which variables are of concern and contribute the most to the NOR while which produce noise. The method will be able to assist in the distinguishing of the critical parameters in soil which are out of NOR as well as in the prevention of unnecessary changes.

Introduction

Soil microorganisms, such as different groups of bacteria, archaea and fungi, are the key drivers of the life support functions (LSF) of soil. They can be considered as the most important determinants in the biotic functioning of soil by playing a dominant role in the cycling of carbon, nitrogen and other compounds. Unfortunately, it is not possible to measure and exploit all soil parameters, since, for example, microbial community structure is often extremely complex and diverse. However, from the wide range of soil parameters that might be addressed, some, which are known to be involved in the most important and sensitive steps of the biogeochemical processes in soil, can be monitored. It was previously pointed out that a suite of different indicators jointly provide a better measure of the status of a complex system such as an organism (Depledge, 1990), a population or a soil (van Straalen, 2002) than a single variable. Thus, the status of a system may be considered as a multivariate property of that system. Therefore, stress or impact can be defined as a deviation from the status, which can be depicted as a baseline state in a multidimensional space. Quantitative assessments of the variation in behavior of the most important and potentially most sensitive microbial groups may lead to the first - to the best of our knowledge - quantitative characterization of the normal operating range (NOR) of a soil as explained in the following.

In natural conditions, the status of a soil will fluctuate without clear consequences, therefore a particular range should be considered as being normal for the system. For instance, Kersting, (1986) defined the 95% confidence space of undisturbed states as the NOR of the system; this allowed for the identification of stress wherever the selected state variables fell outside of the NOR. The definition of a standard of functioning has been instrumental in ecotoxicology, where pollutants can be considered stressors of microbial communities if measured state variables fall outside the NOR or if a shift in the NOR is observed (Medina *et al.* 2007, Schmitt-Jansen and Altenburger, 2005). Nevertheless, the strict definition of NOR has not been developed within an ecological context, and thus it does not consider how the magnitude of the NOR may vary according to the soil studied.

In order to understand the relevance of management- or stressor-induced changes, the natural variation of soil function caused by natural effects needs to be understood better. Mathematical treatment of the data can help us in this. Up to now, there are only few mathematical methods applied in biology which can satisfactorily address the NOR of soil (Dominati *et al.*, 2010; Rutgers at al., 2012; van Wijnen *et al.*, 2012). These were based on a combination of expert opinions and mostly classical soil parameters, such as total carbon or nitrogen levels. These approaches do not consider recent investigations in soil functioning (Pereira e Silva *et al.*, 2012) and, therefore, functional microbial groups as possi-

ble indicators of soil quality are not included. The approach which is described below was selected as the best in term of the quality of obtained results, accuracy, as well as possible applicability by end users. This is achieved by taking into account soil and microbial measurements simultaneously. Parameterization of the model was done by performing data from microcosm experiments as well as by sampling selected soils in natural conditions.

Material and Methods

Soils

The eight soil sites sampled are located in the Netherlands. These fields include four sandy (B, V, D, W) and four clay soils (S, K, G, L). Their characteristics and geographical coordinates are found in Table 9.1. Sampling points were selected to reflect seasonal differences in external parameters, and replicate bulk soil samples were collected four times over an annual cycle in 2009 (April, June, September and November), three times in 2010 (April, June and October), and four times in 2011 (February, April, July and September). Each sample was placed in a plastic bag and thoroughly homogenized before analysis. A 100-g subsample was kept at 4ºC and used for chemical analyses and molecular activities, whereas the remaining soil was kept at –20ºC for subsequent DNA extraction and molecular analysis of bacterial, archaeal, fungal, ammonia oxidizing and nitrogen-fixing community structures and total abundances (see below).

To distinguish an importance of *nitrification-related variables in comparison with the full set of general variables*, soil samples collected in the island of Schiermonnikoog (The Netherlands) was collected in April 2010 following the same scheme as it is described above. The location is 200 meters away from a shoreline facing regular flooding.

Table 9.1. List of soils included in this study.

Sampling Site	Soil type	Land use	North coordinate	East coordinate
Buinen (B)	Sandy loam	Agricultural	52°55′386″	006°49′217″
Valthermond (V)	Sandy loam	Agricultural	52°50′535″	006°55′239″
Droevendaal (D)	Sandy loam	Agricultural	51°59′551″	005°39′608″
Wildekamp (K)	Sandy loam	Grassland	51°59′771″	005°40′157″
Kollumerwaard (K)	Clayey	Agricultural	53°19′507″	006°16′351″
Steenharst (S)	Silt loam	Agricultural	53°15′428″	006°10′189″
Grebedijk (G)	Clayey	Agricultural	51°57′349″	005°38′086″
Lelystad (L)	Clayey	Agricultural	52°32′349″	005°33′601″

Copiotrophic and oligotrophic bacteria

Two samples from each treatment of approximately 1 g were suspended in 4.5 ml water, vortexed for 1 minute and serially diluted. Fifty microliters of suitable dilutions were plated in duplicate on high and low carbon medium for quantification of respectively copiotrophic and oligotrophic bacteria. The high carbon medium contained 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KNO_3, 1.3 g $K_2HPO_4 \cdot {}_3H_2O$, 0.06 g $Ca(NO_3)_2 \cdot {}_4H_2O$, 25 g glucose, 2 g enzymatic casein hydrolysate (Sigma Aldrich Chemie Gmbh, Steinheim, Germany) and 17.0 g Agar no. 3 (Oxoid Limited, Basingstoke, UK) per liter. The low carbon medium was similar but with 1000 fold diluted carbon concentration. After incubation for 60 h on high-C medium (for copiotrophic bacteria) and 14 days on low-C medium (for oligotrophic bacteria), bacterial colonies

Experimental data

For the development of the model, data on measurable key processes (Pereira e Silva *et al.*, 2010; Pereira e Silva *et al.*, 2012), including disturbance-sensitive ones (Pereira e Silva *et al.*, 2012), in soil were used (Table 9.2). The model was validated

Table 9.2. List and description of biotic and abiotic parameters involved in quantitative assessment of soils.

Parameter	Values	
	Sandy soils	Clayey soils
Chemical parameters		
pH	4.70 ± 0.34	6.89 ± 0.89
$N\text{-}NO_3$ (mg kg^{-1})	44.12 ± 0.34	37.29 ± 52.64
$N\text{-}NH_4$ (mg kg^{-1})	27.13 ± 28.82	22.92 ± 21.46
Organic matter (%)	7.26 ± 6.52	4.29 ± 1.47
Nitrification-related biological parameters		
Nitrification ($\mu gN\text{-}NO_2^- + N\text{-}NO_3^-.h^{-1}.gdw^{-1}$)	0.27 ± 0.25	1.10 ± 0.47
Archaeal *amo*A gene abundance (log_{10} copy numbers gdw^{-1})	5.89 ± 0.75	6.43 ± 0.61
Archaeal *amo*A gene diversity (Shannon - DGGE)	2.23 ± 0.45	2.32 ± 0.56
Bacterial *amo*A gene abundance (log_{10} copy numbers gdw^{-1})	6.39 ± 0.77	6.60 ± 0.78
Bacterial *amo*A gene diversity (Shannon - DGGE)	2.53 ± 0.24	2.55 ± 0.21
General biological parameters		
*nos*Z gene abundance (log_{10} copy numbers gdw^{-1})	7.85 ± 0.71	7.87 ± 0.69
Denitrification ($\mu gN\text{-}N_2O.h^{-1}.gdw^{-1}$)	0.69 ± 0.51	1.64 ± 0.91
*nif*H gene abundance (log_{10} copy numbers gdw^{-1})	5.59 ± 0.66	6.41 ± 0.54
*nif*H gene diversity (Shannon - DGGE)	2.81 ± 0.26	2.82 ± 0.28

*Source: (Pereira e Silva *et al.*, 2011; Pereira e Silva *et al.*, 2012)

and tested with experimental data from extra experiments (Pereira e Silva *et al.*, 2012). The detailed description for chemical and biological methods is provided in (Pereira e Silva *et al.*, 2011; Pereira e Silva *et al.*, 2012).

Assessment of disturbances

In order to characterize short-term variations and the influence of different stress factors on changes in the microbial communities in an agricultural soil, a microcosm experiment was carried out. Sandy soil (B) was exposed to two different types of stresses: 1) 30°C for 12 h; addition of water to 100% water holding capacity (WHC) for 12 h; drying to initial 65% water holding capacity and 2) 60°C for 12 h; addition of water till 100% WHC for 12 h; drying till initial 65% WHC. Five parameters were randomly selected (pH, organic matter, copiotrophic and oligotrophic bacteria and 16S DGGE) and were measured after application of the stress to soil.

Quantification and assessment of soils

The NOR is considered as a space of n dimensions, where n is the number of biotic and abiotic variables measured (resulting in a space with 21 dimensions, Table 9.1). All combinations of the variables for non-disturbed sandy and clay soils fall in the "normal operating range" (Fig. 9.1). The NOR was defined as 95% confidence area of states. The distance (1) between a certain state and the center of the NOR represents a quantitative measurement that summarizes the state of the soil (fig. 9.2), taking into account the multivariate nature of the data:

$$distance = \sqrt{(a\ ref\ i - a\ obs\ i)^2 + ... + (a\ ref\ j - a\ obs\ j)^2} \quad (1)$$

Where *a ref i…j* is any parameter which characterizes the center of the NOR, while *a obs i…j* is any parameter indicates an investigated state which is compared with the NOR, distance is a measurement that characterizes how far an investigated state of compared soil is from the NOR. To avoid unequal effect of absolute values of variables, all of them were related to one. All calculations were carried out in MATLAB (Version 11.0, The MathWorks, Inc.).

Results and Discussion

One of the most important aspects in assessments of complex natural systems such as soil is an integration of all appropriate variables into one digit which should be able to characterize its status. Although such integrations will often result in losses of the underlying information, they will still assist us in providing decisions on soil quality and management. Thus, our approach determines the Euclidian distance (*distance*) in multivariate space between a predefined

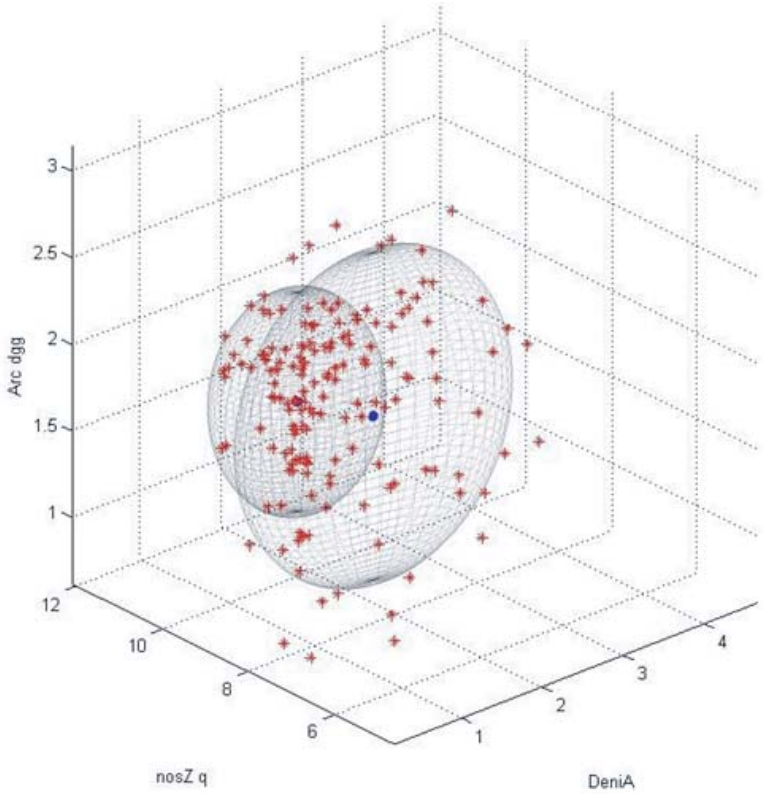

Figure 9.1. The ellipsoids represent edges of the NOR (95% confidence area) of sandy (A) and clay soils for 3 parameters (which are selected merely for visualization) out of 21 used in the calculation of the NOR. Red crosses are observed values.

reference state - which is considered as the center of multidimensional NOR - and an investigated state, in which every dimension is represented by the most descriptive/explicative biotic and abiotic soil characteristic.

During the analysis, an investigated state might be in two conditions. The first one occurs if all measured variables fall in the multidimensional space of the NOR; in this case the investigated state is considered to be similar to the NOR reference state in which no undefined stresses or disturbances are present. On the other hand, if one or more variables are out of the NOR, this investigated state might be under the risk of unexpected changes such as erosion, decrease of important nutrients, etc. In this case, the *distance* between the multidimensional edge of the NOR and the investigated state is >0 (Fig. 9.2). Moreover, based on results as to the influence of disturbances, a longer *distance* will indicate the presence of more risky or pronounced changes (see example below). It is also important to distinguish which variables contribute the most

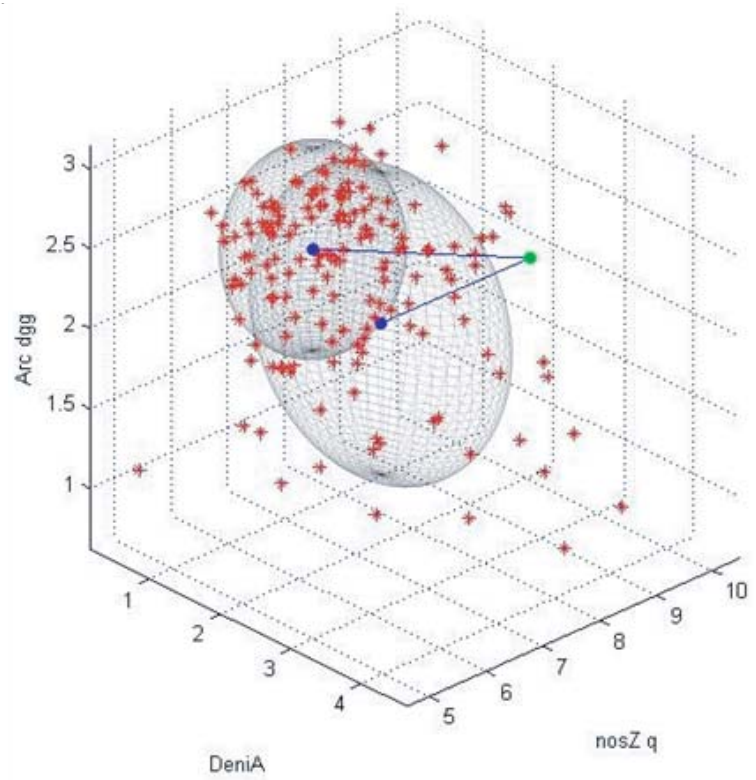

Figure 9.2. The ellipsoids represent edges of the NOR (95% confidence area) of sandy (A) and clay soils for 3 parameters (which are selected for visualization) out of 21 used in the calculation of the NOR. Red crosses are observed values. The distance between an investigated state (green dot) and the center of the NOR (blue dot) for sandy and clay soils represents a quantitative measurement that summarizes the state of the soil.

to placing the investigated state out of the NOR. Soil processes and parameters related to such variables are to be considered as of most concern (Fig. 9.3).

While testing integrated sets of data, it became clear that some reference soils for the NOR parameterization were significantly different from other ones and could not be united within one multidimensional space. We thus tested whether two (or more) groups could be discerned. An analysis of all soils that were tested indicated that the most efficient separation was by soil texture. Thus, two pre-defined NOR states were considered, the first state was for sandy soils, while the second was for clay soils (Fig. 9.1; Fig. 9.2).

The advantage of the proposed approach is that not all of the selected 21 variables have to be measured for an investigated soil to compare it with the NOR established for that soil type. Thus, we performed Monte-Carlo analyses (100,000 calculations each) and showed that 7 randomly selected variables

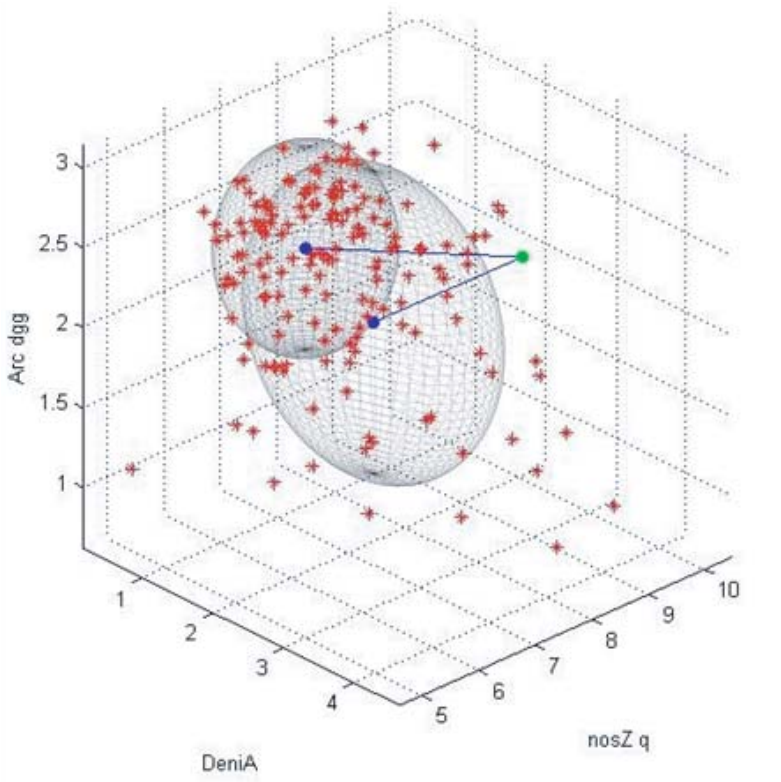

Figure 9.2. The ellipsoids represent edges of the NOR (95% confidence area) of sandy (A) and clay soils for 3 parameters (which are selected for visualization) out of 21 used in the calculation of the NOR. Red crosses are observed values. The distance between an investigated state (green dot) and the center of the NOR (blue dot) for sandy and clay soils represents a quantitative measurement that summarizes the state of the soil.

would be enough to distinguish that an investigated soil is out of the NOR at a probability of 97%. Moreover, in many cases, 5 random variables would be enough as well (probability 90%). This obviously gives equal weight to all these parameters and does not take into account that some may be much more important than others. However, most likely our ability to distinguish that an investigated soil is under stress and outside of the NOR is even higher since the parameters to be measured are usually selected according to soil assessment needs.

To test the proposed approach as well as the sufficiency of just a few variables to distinguish soils under stress and outside of normality, an experiment with two types of stress was carried out. Temperature increases (to 30°C and 60°C for 2 stress treatments, respectively), followed by 12h flood (100% WHC) were meant to simulate possible disturbances in natural conditions. Such types

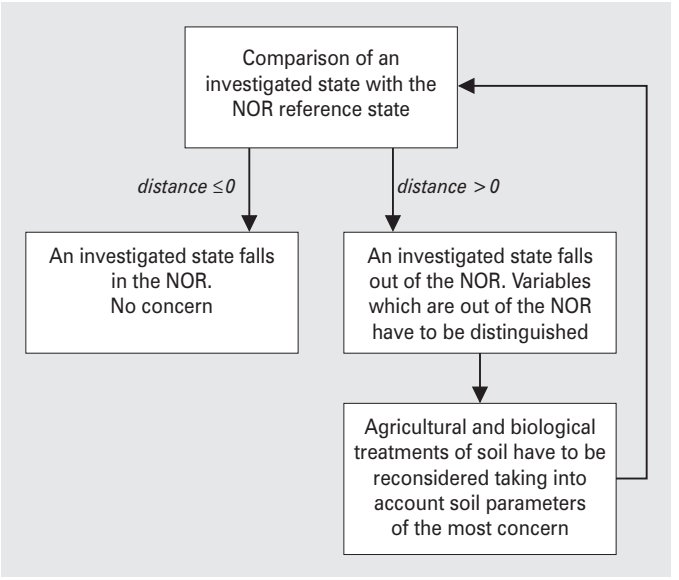

Figure 9.3. Comparison of an investigated state with the NOR reference state.

of stress do not allow predicting which soil parameters would be under strong impact and, most likely, fall out of the NOR. Therefore, five randomly selected variables were measured (pH, organic matter, copiotrophic and oligotrophic bacteria and 16S DGGE). While all variables for a control treatment fell within the NOR (*distance* ≤ 0), two other treatments under stress had several variables that fell outside of the NOR (namely, copiotrophic and oligotrophic bacteria and 16S DGGE), resulting in a *distance* > 0. Interestingly, the more pronounced the applied stress was (30 versus 60 C), the further the state of the stressed soil was from the NOR (11.9 for stress A vs 25.0 for stress B) (Fig. 9.4). Moreover, only the biologically related variables were sensitive enough to show clear responses. Therefore, the proposed approach does not only highlight if the status of an investigated soil is outside of its NOR, but also provides information about severity of the state.

Although our approach does not have direct predictive capacities, it clearly highlights which processes should be carefully checked. The reason is that, next to classical soil parameters such as pH or organic matter, a significant part of the variables that are included represents functional microbial groups responsible for turnover of nutrients in soil (Table 9.1). The microbial communities in soils are diverse and the function of each member is often not well known. Therefore, it is important to play a focus on the selected key function and the microbial groups involved. Thus, while all fundamental soil parameters might

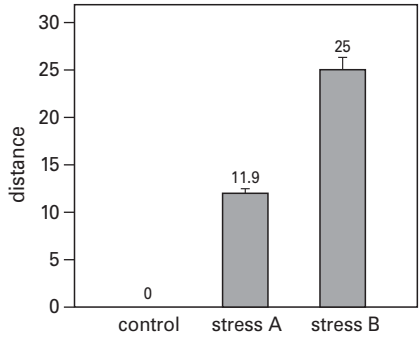

Figure 9.4. The influence of two types of stress (stress A: 30°C for 12 h; addition of water till 100% water holding capacity for 12 h; stress B: 60°C for 12 h; addition of water till 100% water holding capacity for 12 h, in both cases drying till initial 65% water holding capacity) on the distance which characterizes how far an investigated state of soil is from the NOR.

be still within the NOR at the current stage (e.g. Norg), significant decreases of e.g. ammonium oxidizers could suggest that the nitrogen cycle is out of balance, which most likely will lead to unpredictable changes in the nitrogen-related nutrients as well.

Thus, a multidimensional state of NOR based on only nitrification-related variables (Table 9.1) would be able to distinguish more pronounced deviations from the NOR when compared to the NOR based on the full set of general parameters (Table 9.1, Chemical parameters and General biological parameters). Indeed, the *distance* observed between a soil under stress (*Schiermonnikoog* soil, under constant flooding) to the NOR based on nitrification-related parameters was much higher than the corresponding value between the NOR part based on the other parameters (90.77 vs 4.03, respectively). Interestingly, the thus defined *distance* was also higher in comparison with the NOR based on all available variables (Table 9.1., all variables). These results support the hypothesis that the focus on sensitive parameters like offered by the nitrification-related ones, is an appropriate direction to follow since the chances to distinguish soils under various disturbances are expected to be higher.

Estimating the parameters that define the NOR using such proxies for subsets of the indigenous microbial communities in soils will thus be at the core of a sensitive method that distinguishes the influence of possible soil disturbances (e.g. usage of GMO, herbicides or fertilizers), putting the soil system outside of its normal range. Moreover, the tool can assist in distinguishing the critical parameters in soil which are out of NOR as well as in the prevention of unnecessary changes. In most of the cases, only 5-7 variables from 21 available are enough to distinguish soils under stress conditions, especially if several of these

variables are pre-selected from the nitrification-related biological parameters (Table 9.1). For each soil type (clay vs sandy soil; low pH vs high pH soil etc.), the multidimensional state of the NOR may subsequently be re-parameterized, enabling a comparison of the data obtained on presumably healthy and balanced versus disturbed soils under various management practices. Finally, knowing the biochemical soil characteristics and those procedures that lead to a more stable and balanced soil would directly point to ways to preserve soil health and stability.

Acknowledgements

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Chapter 10

Discussion and outlook

Michele C. Pereira e Silva

Understanding the fluctuations in soil microbial communities and their function

The biodiversity (number of different organism types and their relative abundance) of the living soil is uncountable. The number of bacterial types per gram of soil may reach 8.3 million in pristine soil (Gans *et al.*, 2005; Schloss and Handelsman, 2006). Moreover, soil microorganisms are known as key players in a large number of important ecosystem processes, e.g., nitrogen cycling (Kowalchuk and Stephen, 2001), carbon cycling (Hogberg *et al.*, 2001) and soil formation (Rilling and Mummey 2006). Thus, soil microorganisms are important in maintaining the quality of both natural and agriculturally managed soil systems.

The organisms in soil are also highly responsive to environmental influences, such as those incurred by abiotic and biotic factors. They also change in response to agricultural practices (van Overbeek and van Elsas, 2008) such as plowing (Buckley *et al.*, 2001; Clegg *et al.*, 2003) (Figure 10.1). Furthermore, soil type (Bossio *et al.*, 1998; Girvan *et al.*, 2003) and soil pH (Fierer and Jackson 2006; Lauber *et al.*, 2009) are major abiotic factors influencing soil microbial communities. Nevertheless, the extent to which these factors affect soil microbial communities remains unclear. In the previous chapters, I discussed the drivers of the fluctuations in abundance, structure and composition of soil microorganisms, and in this synthesis I will try to connect the main findings from the perspective of how soil dynamics is linked to soil stability and "soil normality", which are key concepts in the search for the normal operating range of soil.

Fluctuations in soil nitrogen cycling communities and related processes

In this thesis, I have characterized the dynamic changes in the abundances and structures of soil microbial communities, focusing on the bacterial, archaeal and fungal communities (**Chapter 2**), and more specifically on nitrogen fixers (**Chapters 3 and 5**) and ammonia oxidizers (**Chapters 4 and 6**), across eight different representative Dutch soils. In **Chapter 2**, I analyzed the temporal variations and spatial responses of soil bacterial, archaeal and fungal communities to abiotic parameters, taking into account data from a 3-year sampling period. I found that, although the structure and abundance of bacterial and fungal communities showed some fluctuations over time, they didn't significantly correlate with the soil parameters measured. On the other hand, and surprisingly, I observed a higher sensitivity of archaeal communities to soil parameters, suggesting that archaea, rather than bacteria or fungi, are mostly driven by environmental factors. It might be that for bacterial and fungal communities, biotic parameters (e.g., the interaction between species) are more important driving factors. Indeed, Zinger and coworkers (2011) investigated soil microbial communities at eleven contrasting habitat types in an alpine landscape, and found that bacterial and fungal assemblages were mainly affected by plant-soil inter-

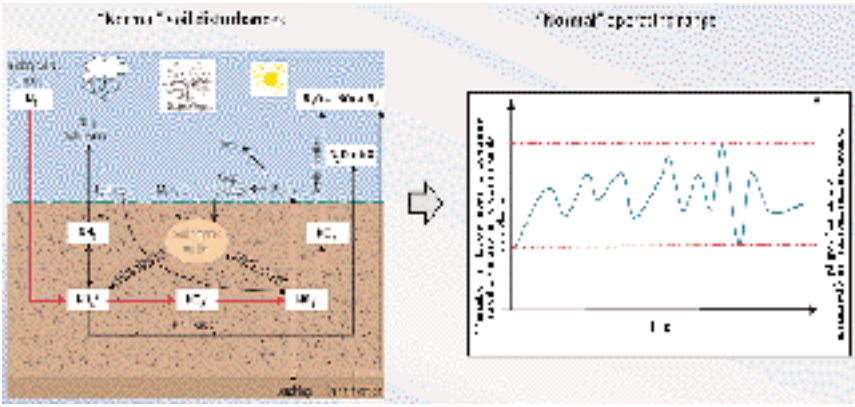

Figure 10.1. Representation of the nitrogen cycling microbial community and processes and how they can be affected by natural (seasonal) and anthropogenic (addition of fertilizers) sources of variation. The integration of these factors leads the microbial community and associated processes to fluctuate over time, establishing the so-called normal operating range (NOR). The processes studied in this thesis are highlighted in red.

actions, mainly through variations in ecological associations. On the other hand, the structure of crenarchaeal communities was explained mainly by environmental conditions, i.e. soil pH.

Moreover, I evaluated the amplitude of the natural variation of nitrogen fixers and ammonia oxidizers. Concerning the nitrogen-fixing community, I observed a significant influence of time / season, as reported by Mergel *et al.* (2001). For instance, the *nif*H gene abundance was significantly lower in April of 2009 and 2010 than in November 2009 or October 2010 (**Chapters 3 and 5**). These results could be explained by variations in temperature, soil moisture, availability of carbon (Hsu and Buckley, 2009) or a combination of these factors. In **Chapter 3** we reported a highly dynamic nitrogen-fixing community, and turnover rates between 60 to 65% in community composition were estimated. These results were confirmed in **Chapter 5** through pyrosequencing based on the *nif*H gene, where, strikingly, in some cases a different community was present at each sampling time. It remains unknown, however, how these changes in community structure and size over time affect the functioning.

The ammonia oxidizing communities also showed significant fluctuations over time and so did the potential nitrification rates (**Chapter 4**). The variability of the AOA and AOB community structures was around 50% for the AOA and around 60% for the AOB over the year 2009. The abundance of these groups also varied significantly over time, being usually lower at the beginning of the season (e.g. April) and higher at the end (October and November). A more complex situation was observed when the nitrification process was evaluated.

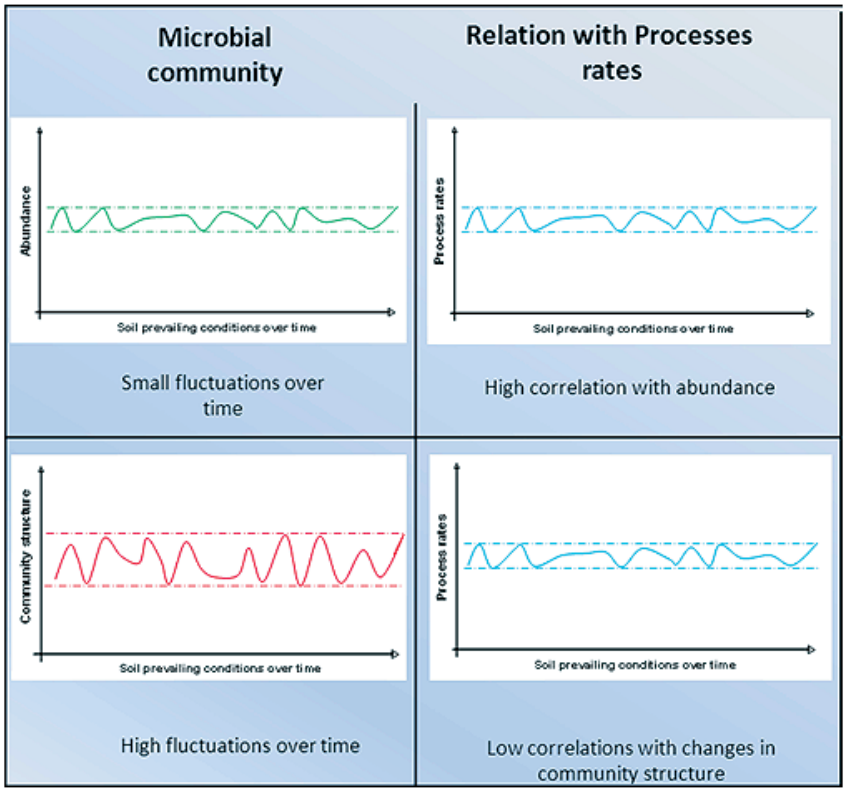

Figure 10.2. Relationship between process rates, gene abundance and community structure over time. Abundance is highly correlated with the process, whereas community structure is not. This does not mean that the fluctuations of abundance and activity have the same magnitude. In general AOA abundance fluctuates more over time, for instance, than do nitrification. Although community structure fluctuates greatly, changes in process rates are low and cannot be predicted by analyzing community structures.

Nitrification rates fluctuated in the clayey soils but they were quite stable in the sandy soils. Moreover it was possible to predict changes in nitrification to a higher extent by quantifying AOA gene abundances instead of analyzing the community structure of the AOA or AOB. Thus, by measuring abundances, a fair relationship with the process can be achieved (Figure 10.2).

Significance of the fluctuations in soil microbial communities and processes for soil normality

As observed in the foregoing, rather than being constant in time and space, I found a strong temporal variability in the nitrogen fixers (**Chapters 3 and 5**),

ammonia oxidizers (**Chapters 4 and 6**) and to a lesser extent in the total bacterial, fungal and archaeal communities in soil. Such changes are likely to cause alterations in process rates, as biological communities and ecological processes are intimately associated. While the functional significance of the complex soil microbiota to ecosystem functioning is well established, the relationship between species diversity, functional diversity and functional composition with the rate and intensity of an ecological process, in a fluctuating environment, is still under debate. These fluctuations and recurrent natural disturbances can generate novel assemblages of species (Neilson *et al.* 2005), which might lead to changes in community structure (Frostegard *et al.*, 1996; Witter *et al.*, 2000; Boivin *et al.*, 2002; 2005). This adaptation to seasonal fluctuations might explain the lack of response of soil bacteria and fungi in **Chapter 2**. Moreover, I have observed that, for instance, total archaea, nitrogen fixers and ammonia oxidizers have different sensitivities to particular environmental parameters. The higher sensitivity of soil archaeal communities to seasonal fluctuations was a key finding, as important and sensitive soil processes are carried out by some members of this group, e.g. nitrification by ammonia oxidizing archaea and methane production by methanogenic archaea.

Furthermore, not all processes will be affected to the same extent. In general, redundant processes, i.e. those carried out by many species, e.g. respiration, denitrification and nitrogen mineralization, are expected to be less affected by soil disturbances (Wertz *et al.*, 2007). Even very high fluctuations in soil communities will likely not influence those processes. Indeed, I quantified the abundance of the nosZ gene, which encodes nitrous oxide reductase, the enzyme responsible for the last step of denitrification, five times from November 2009 to October 2010. I also measured the potential denitrifying activity (DEA) (data not shown). I did not observe any significant seasonal fluctuation or correlation between abundance and processes rates, indicating that this community is not as responsive as nitrogen fixers or ammonia oxidizers, to say at least. On the other hand, narrow processes, e.g. nitrification, are carried out by only few specialized species. The first step of nitrification, the oxidation of ammonium to nitrite via hydroxyl amine, is performed only by ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaeal (AOA), and is considered as very sensitive to disturbances (Bruisma *et al.*, 2003; Kowalchuk *et al.*, 2003). The fact that ammonia oxidation is known as the rate-limiting step of nitrification is the reason why I focused on these communities and not on nitrite oxidizing bacteria (NOB), which are also important and are responsible for the second step in nitrification, i.e. the oxidation of nitrite to nitrate. Nevertheless, nitrite-oxidizing bacteria have been shown to respond to management practices (Freitag *et al.*, 2005; Attard *et al.*, 2010), and can eventually become limiting in disturbed soils (Roux-Michollet *et al.*, 2008; Gelfand and Yakir, 2008). The high sensitivity of the ammonia oxidizing communities to seasonality was clear from **Chapters**

4 and 6, where almost completely different AOA communities were observed throughout the season (**Chapter 6**).

Considering the processes studied in this thesis, nitrogen fixation and nitrification, I found that the seasonal fluctuations of the microbial communities and related processes are soil type as well as process-dependent. It has been proposed that soils with high clay content provide better conditions for species to coexist (Tiedje *et al.*, 2001) due to spatial isolation which reduces competition between species. I observed that abundance as well as the composition of AOA in clayey soils fluctuated more than in sandy soils. The same results were observed for nitrification rates (which were correlated to AOA abundance to a higher extent than to AOB abundance), with sandy soils showing less fluctuation over time than clayey soils. These counterintuitive results suggest that agricultural sandy soil might represent more steady systems when considering nitrification. The results obtained with *nif*H gene amplification suggested a different picture, with fluctuations in abundance and composition being higher in sandy soils, as compared to clayey ones.

Influence of soil parameters, in particular pH and texture, on microbial communities associated with nitrogen cycling

Several parameters are known to influence soil microbial communities. Among them, soil pH is known as a main driver of soil microbial community composition (Fierer and Jackson, 2006; Hartman *et al.*, 2008; Jenkins *et al.*, 2009; Lauber *et al.*, 2009). However, clay minerals influence the physicochemical properties of soil, and might thus affect the dynamics of soil microorganisms. As clay content is intrinsically associated with soil pH, with clayey soils usually having higher pH, it is difficult to unravel which is the driver of changes in microbial communities. In **Chapter 3**, I found that the amplitude of variation in the abundance of the *nif*H gene was higher in clayey soils, which had higher pH, than in sandy soils, with lower pH. The amplitude of variation of AOB community structure was also higher in the clayey soils, whereas for AOA the amplitude of variation was higher in the sandy soils (**Chapter 4**). The observed fluctuations were hypothesized to be soil type dependent, although an associated effect of pH could not be ruled out at that point.

In **Chapter 5**, through pyrosequencing based on the *nif*H gene, I was able to identify several soil type specific species, for instance *Paenibacillus* and *Thermochromatium* species in the sandy soils, and *Azoarcus* and *Burkholderia* species in the clay soils. I also observed the widespread presence of *Bradyrhizobium* spp. in all soils, which fluctuated seasonally. The variation in community composition of these groups was also influenced by soil pH and clay content. A clear effect was observed on the composition of AOA analyzed by pyrosequencing (**Chapter 6**), which formed two major clusters, an acidic-alkalinophilic and an alkalinophilic one. The latter seemed to correlate more with changes in

potential nitrification. In **Chapter 7**, I aimed to understand the separate effects of clay content and soil pH on the abundances of the aforementioned functional groups (AOA, AOB and nitrogen fixers).

I observed a strong effect of both the addition of montmorillonite, a component of clay soils, and altered soil pH, on both the abundance and activity of nitrogen-cycling microorganisms, which was group-dependent. The abundance of the AOB decreased significantly by the addition of clay. I speculate in this chapter that the AOB populations inherent to the sandy soil used as control might not have survived in very clayey environments, which might be too hostile for these communities. On the other hand, the abundances of AOA and nitrogen fixers were not affected by clay, suggesting that AOA and nitrogen fixers are less sensitive to soil texture compared to AOB. I also perceived the significance of time on the abovementioned effects. More specifically, the influence of clay content and pH on the abundances varied depending on the sampling time, e.g. how established the community was in the soil. This was very clear when analyzing how pH influenced the abundance of AOA and AOB, and finally in the nitrification rates. At the beginning of the experiment, when the soil community was not well adapted, AOA seemed to be responsible for the nitrification activity measured. As we went further in the succession time, there was an inversion and AOB, but not AOA, were significantly and highly correlated with nitrification activity. It could be that AOA might drive nitrification in soils that have been recently disturbed, whereas AOB might be more relevant in less disturbed ecosystems.

Overall, in **Chapter 7**, I observed a significant increase in abundance with increasing pH. Moreover, for AOB, the effect of the increase of clay content was deleterious to the community, whereas for nitrogen fixers (until 45% clay) and AOA it was not. Considering that I always observed higher abundances of these groups in the four clayey soils collected during this study, I can conclude that these higher abundances more likely reflected their higher pH, as previously suggested (Fierer and Jackson, 2006; Hartman *et al.*, 2008; Jenkins *et al.*, 2009; Lauber *et al.*, 2009), and not their texture. This finding is very important, and provided evidence that, in order to study the natural fluctuations of soil communities in terms of its normality, an initial classification by soil pH and not texture, is primordial.

The normal operating range of soil functioning

Recently, there has been a change in the perception of the importance of soil for ecosystem functioning and the need to improve soil functions, sustaining biological productivity, promoting environmental quality and maintaining plant and animal health (Doran and Zeiss 2000). Agricultural practices and changes in management regime can have serious and long-lasting effects on soil microbial communities (Clegg *et al.*, 2003; Salles *et al.*, 2006). Also, the introduction of

new cultivars (GM or non-GM), planting time or pest controls (Lilley *et al.*, 2006) will influence agricultural productivity. Despite the importance of these factors in shaping soil diversity, little is known about their long-term temporal patterns.

In order to truly understand these "normal" fluctuations, I started generating a large data set with data of abundance (qPCR), structure (PCR-DGGE), composition (clone libraries and pyrosequencing) and functioning (NEA) of the microbial community associated with nitrogen cycling. This data set is included in the so-called normal operating range (NOR) of soil functioning, and establishes a NOR for soil nitrogen oxidation. Further, it establishes how soil microbial community size, structure and composition vary under "normal" sources of variation and, most importantly, how this variation relates to process rates in order for the ecosystems to be considered "healthy". Moreover, by identifying the key drivers of biological changes, we can single out the most probable factors that would put the system outside the NOR, which function would be affected, and on what basis the NOR should be established, e.g. one for all soils, different NORs per soil type, per pH, type, etc.

As observed from this thesis, an initial classification should be based on the pH of the soil under evaluation, as pH was identified as a major driver of changes in abundance and composition of nitrogen cycling communities. Furthermore, a careful study was done across this thesis, in which I came to the conclusion that particular soil microbial groups should be selected depending on the soil pH. It is likely that further classifications should also be performed, such as by type of system (agricultural or forest), or even by soil type (silty or high-organic). It is also plausible that land use and/or management practices are included into these major NOR types. In the future, the NOR (representing the natural fluctuations) might provide a background against which to compare any impacts on soil functioning. Thus, the soil NOR turns into a tool for the educated judgment of soil normality, allowing a clear visualization of systems under disturbance. More detailed and rigorous experiments are necessary to confirm these contentions and to evaluate the real impact of stress on soil functioning and the resilience of the system, which cannot be inferred from the NOR at this stage.

Can we use microorganisms involved in nitrogen cycling as proxies of environmental disturbances?

It is known that microbial composition and function are sensitive to variability and extremes in soil conditions (Stark and Firestone, 1996; Gulledge and Schimel, 1998; Fierer *et al.*, 2006). As discussed above, soil microbial communities harbor different species with different sensitivities to natural and/or anthropogenic disturbances. These disturbances can generate novel assemblages of species (Neilson *et al.* 2005), as not all species respond in the same way

and species resistant to a particular dominant or recurring stress can thus become abundant. This will lead to changes in community structure and tolerance (Frostegard *et al.*, 1996; Witter *et al.*, 2000; Boivin *et al.*, 2002; 2005). Moreover, although some disturbances can completely destroy a community, recurrent disturbances may drive microbial community towards an enhanced (Dornelas *et al.*, 2011) or diminished diversity. Both situations might have an impact on ecosystem processes.

As discussed previously, I have found that nitrogen fixers as well as ammonia oxidizers represent very dynamic communities in soil, being sensitive to fluctuations associated with season and agricultural practices. Although unfortunately I did not measure the rates of nitrogen fixation in the soil samples, it has been proposed that the abundance of *nif*H genes correlates significantly with the rates of nitrogen fixation (Wakelin *et al.* 2005, 2010; Huang *et al.*, 2011). I also observed that fluctuations in AOA abundance were lower in sandy soils and those of AOB were lower in clayey soils. Moreover, the abundance of *nif*H also fluctuated less in clayey soils. These results might indicate that for agricultural sandy soils, nitrification-based parameters might represent fair indicators of disturbances, whereas in clayey soils other parameters than nitrification-based ones (e.g. nitrogen fixers or other sensitive groups) might be better suited. These findings have important consequences for what is considered to be "normal" for a soil and how to detect that a system is under "stress". Clearly, normality will likely vary depending on the soil system being evaluated, e.g. whether agricultural or forest, with soil type (and all its inherent features, e.g. pH) and possible management regimen. As discussed above, even the indicators used will change the manner we perceive soil normality and stability.

From these results, I conclude that these functional groups can indeed be used to monitor soil disturbances, as they have been proven to be sensitive enough. However some issues need to be taken into account. First, their seasonal "stability" (meaning their fluctuations) is directly linked to soil type, more specifically to soil pH, with more stable systems having lower-amplitude variations after having been disturbed (Ives and Carpenter 2011). Second, the stability of these communities to (non-seasonal) fluctuations and their potential use as indicators in such situations is still unclear. Furthermore, soil has an inherent potential to resist (resistance) and recover from (resilience) environmental stresses (Griffiths *et al.*, 2000; Tobor-Kaplon *et al.*, 2005), features that are extremely important as they are closely related to the limits of the NOR and the "normality" of a particular system (Figure 10.3). For instance, the AOB community structure from soils contaminated with heavy metals has been reported as stable even after 13 weeks of phytoremediation with poplar, a fast growing tree able to remove heavy metals from terrestrial environments (Frey *et al.*, 2008), suggesting that AOB might be a resistant community. Indeed, AOA have been found to be more responsive to fungicide use than AOB in a litter soil cover, as

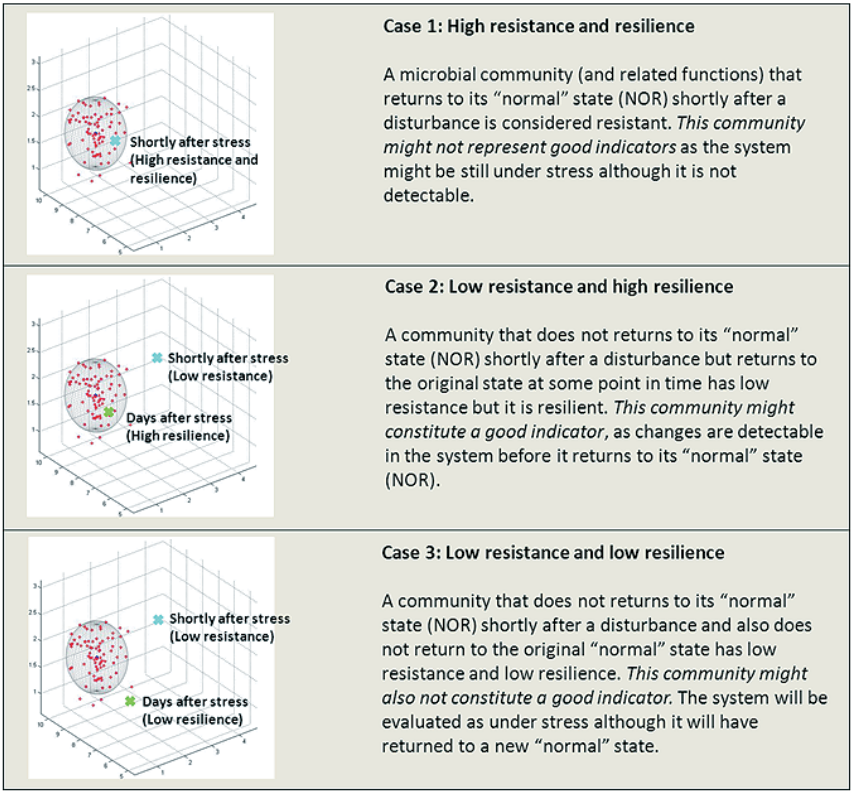

Figure 10.3. Possible behavior of soil microbial community (and related functions) after a non-seasonal disturbance and implications for the selection of bioindicators and the NOR of soil functioning. Here the ellipsoid represents the borders of the NOR and the red crosses are the observed values used to characterize a given system.

measured by RNA-based DGGE profiles (Puglisi *et al.*, 2012). The authors observed an immediate change in AOA community structure till day 56 after fungicide use, whereas changes in AOB community structure were only detected from day 56 onwards.

The use of nitrogen fixers, AOA or AOB as indicators might also depend on the type of stress and how adapted the original soil community is. Moreover, Mertens and colleagues (2006) demonstrated that AOB populations were sensitive to increasing Zn concentrations in artificially contaminated soils, whereas AOB populations from long-term contaminated soil samples were able to tolerate higher Zn concentrations than AOB populations from uncontaminated soil samples. This points to a role of the physical placement of the cells in a soil, which is bound to be different between recently inoculated and long-term soils.

Yet, microcosm experiments are needed to validate the potential use of these groups as indicators of disturbances, taking into account their resistance and resilience to disturbances.

Conclusion

Soils are major contributors to global nutrient cycling processes, which are indispensable for the healthy functioning of our ecosystems. Given the presence of multiple functions in soil ecosystems, we propose the establishment of a NOR on the basis of multiple parameters of the soil. Choosing the parameters that will allow the establishment of the NOR is an important yet difficult task. It might be that, in the light of its dynamic nature, functions and interactions, the soil microbiota may remain a puzzle for a long time. In this thesis, I have discussed how the normal sources of variations interact with each other and how this is reflected in different aspects of soil microbial communities and function, by integrating microbial community dynamics to responses to natural fluctuations, a first step to establish the NOR of soils. I have also identified the basis on which to define the NOR for agricultural soils. Although it is likely that one NOR for all soils is an impracticable concept, I have shown that, by focusing on sensitive and lowly redundant biological parameters, in particular key genes involved in biogeochemical cycles (amoA and *nif*H), a functional NOR can be developed, providing a best picture of what is going on in the soil systems.

Future directions

In my attempt to establish the NOR of soil functioning, I have tried to get insight into how structure, abundance and function of soil nitrogen cycling communities fluctuate, which are the possible drivers of those changes, and what these fluctuations may mean for ecosystem functioning and soil stability. However, some remarks have to be made. There are always constraints when studying soil microbial communities, starting with the nucleic acid extraction itself, which can be biased towards specific groups depending on the method used (Fröstegard *et al.*, 1999; Özgul *et al.*, 2010). Furthermore, fingerprinting methods can misrepresent the soil total diversity, as one single band does not always represent a single strain (Sekiguchi *et al.*, 2001).

The diversity of soil microbial communities is enormous, and therefore it is practically impossible to monitor all organisms and functions in soil. In this thesis, I have selected two steps of the nitrogen cycle, and I have studied how the related communities fluctuate under field conditions, to evaluate their role as proxies of soil disturbances. I found that it is unlikely that a normal operating

range of soil functioning can be established based on one single indicator group, as their limits of variation, their "normality", will be different in different systems and soil types, to say the least. These limits may even change after a disturbance, where a new stable state might be reached.

Finally, I propose two approaches. The first one involves a pre-classification of soils that are intended to be evaluated along soil pH, and then the use of the most suited group of microorganisms for that pH class as a proxy. From this study, I suggest for instance the use of AOA in soils with low pH and sandy texture and nitrogen fixers in soils with high pH and clayey texture. In the second approach no pre-classification of soils is necessary and all parameters related to the selected process are considered. One could even speculate on the possibility of creating specific soil system databases, not only for agricultural but for other soil ecosystems as well, containing few to hundreds of variables (physical, chemical and microbiological) and using these databases in mathematical models, like the one proposed in this thesis (Chapter 8). Such an approach would certainly facilitate and greatly influence soil quality assessments. However, such an approach is based on ideas and hypotheses that still need rigorous testing on larger number of agricultural soils.

Nederlandse samenvatting

English Summary

Nederlandse samenvatting

De bodem is een zeer heterogeen en dynamisch system voor de micro-organismen die erin leven. De biologische diversiteit van de levende bodem is enorm hoog, en schattingen van de aantallen bacterietypen per gram grond belopen tot aan een miljoen. Deze micro-organismen kunnen sterk reageren op, en fluctueren met, bepaalde omgevingsfactoren (verstoringen), zoals de abiotische factoren temperatuur, pH, vochtgehalte en structuur of textuur van de bodem alsmede de biotische factoren samenstelling en diversiteit van de aanwezige microbiele gemeenschap. Genoemde fluctuaties kunnen de dynamiek en activiteit van de bodemorganismen en hun interacties aantasten. Dit kan, samengevat, de normale "operating range" (NOR) van de bodem bepalen. Normaliteit is hier gedefinieerd als omvattend de normale fluctuaties in functie als reactie op alle omstandigheden die in de bodem kunnen voorkomen, via natuurlijke of (normale) anthropogene weg. Een beschouwing van de biogeochemische cycli van de bodem leerde dat de stikstofcyclus sterk beinvloedbaar is. Zowel symbiotische stikstofbinding als nitrificatie zijn voorgesteld als typische processen die gevoelig zijn voor verstoringen. Deze processen zouden derhalve goed kunnen worden aangewend als indicatoren van stresscondities in de bodem. In het licht van deze argumentatie is in dit proefschrift de abundantie, structuur (gemeenschapssamenstelling) en functie van micro-organismen die betrokken zijn bij bepaalde stikstofcyclusprocessen gevolgd in een reeks geselecteerde Nederlandse bodems. Hoofdstukken 1 en 2 introduceren het onderwerp in theoretische zin. In hoofdstukken 3 en 5 zijn de fluctuaties in de structuren (samenstellingen) van de stikstofbindende micro-organismen beschreven. In deze analyses werd gevonden dat verschillende bodemtypen onderscheiden konden worden op basis van deze structuren. Met name was de bestudeerde microbiota in bodems met hoger kleigehalte (kleiachtige bodems) diverser en ook waren de amplitudes van de fluctuaties in de tijd hoger dan die in bodems met een lager kleigehalte (zandige bodems). Daarnaast waren bodemfactoren zoals het gehalte aan ammonium, pH en textuur sterk gecorreleerd met de variaties in de omvang, diversiteit en structuur van de stikstofbindende microbiele gemeenschappen. Dit gaf aan dat stikstofbinders gevoelig zijn voor de abiotische parameters van de bodem, hetgeen hen geschikt maakt als mogelijke indicatoren van verstoringen in de bodem.

In hoofdstukken 4 en 6 is de dynamiek van de ammoniumoxideerders bestudeerd, alsmede de effecten van abiotische factoren in de bodem op de omvang, structuur en diversiteit van deze microbiele gemeenschappen. De verkregen gegevens lieten zien dat bodem pH en bodemtype belangrijke factoren waren die de omvang en structuur van de archaeale (AOA) en bacteriele (AOB) ammoniumoxideerders, naast hun functie, bepalen. Deze gegevens kunnen dienen als de basis waarop de NOR voor nitrificatie in (landbouw) bodems

bepaald kan worden. Omdat de parameters bodemtype en bodem pH intrinsiek aan elkaar gekoppeld zijn, is in hoofdstuk 7 een microcosmosexperiment opgezet waarin de invloeden van bodemtextuur en bodem pH op de abundantie en functie van zowel stikstofbinders als ammoniumoxideerders apart zijn bepaald. De resultaten gaven aan dat de AOA betrokken zijn bij nitrificatie in bodems die recent verstoord zijn, terwijl AOB relevanter zouden kunnen zijn in onverstoorde bodems. Voorts toonden de resultaten aan dat de hogere abundantie van stikstofcyclerende micro-organismen in kleiachtige bodems waarschijnlijk relateren aan de hogere pH in deze bodems en niet aan het textuurtype. Tenslotte wordt in hoofdstuk 9 een wiskundig model ter beschrijving van een kwantitatieve aanpak van de NOR van bodemfunctioneren voorgesteld. Deze modelmatige aanpak zal van belang zijn bij toekomstige bepalingen van de kwaliteit van bodems.

English Summary

Soil represents a highly heterogeneous and dynamic environment for its microbiota, a mixture of different constituents. The biodiversity of the living soil is uncountable, with estimations in the order of million bacterial types in a gram of soil. These microorganisms are known as highly responsive to environmental influences (disturbances), such as those incurred by abiotic (temperature, pH, soil moisture and soil structural or textural type) and biotic factors (the composition and diversity of the microbial community) and will fluctuate in response to these factors. Such fluctuations may affect the dynamics and activities of soil organisms and the interactions between them, and taken together, will define the normal operating range of soils, with normality including the expected fluctuations in function in response to all conditions that occur in a particular soil system, either naturally or due to common anthropogenic influences. When comparing the impact of disturbances on biogeochemical cycles, the nitrogen cycle is strongly influenced, and both symbiotic nitrogen fixation and nitrification have been advocated as sensitive to disturbances, working well as indicators of stress conditions in soil. Therefore, in this thesis the natural fluctuation in abundance, structure and function of nitrogen cycling microorganisms was investigated across a range of selected Dutch soils. In chapters 3 and 5 the structure and dynamic changes in the composition of nitrogen-fixing microorganisms were investigated. From these chapters we observed that different soil types could be discriminated based on this community, where soils with higher clay content (clayey soils) were more diverse and fluctuated more over time than soils with lower clay content (sandy soils). Moreover, soil characteristics such as ammonium content, pH and texture strongly correlated with the variations observed in the diversity, size and structure of nitrogen fixing communities, suggesting that nitrogen-fixing microorganisms are sensitive to abiotic parameters and could represent potential indicators of soil disturbances.

In chapters 4 and 6 the dynamics of ammonia oxidizers were studied and the effects of soil abiotic parameters on the abundance, structure and function of these communities were addressed. The results indicated that soil pH and soil type were also main factors that influenced the size and structure of the archaeal (AOA) and bacterial (AOB) ammonia oxidizers, as well as their function. From this chapter it was observed that AOA and AOB have different amplitudes of variation in terms of both abundance and structure. The data might be used as a basis when defining the NOR of nitrification in agricultural soils. As soil type and soil pH are parameters intrinsically related, in chapter 7 a microcosm experiment was set up to evaluate the influence of soil texture and soil pH on the abundance and function of nitrogen fixers and ammonia oxidizers. The results suggested that AOA might drive nitrification in soils that have been recently disturbed, whereas AOB might be more relevant in pristine

ecosystems. The results also indicated that the higher abundance of nitrogen cycling microorganisms observed in clayey soils are likely a reflection of their higher pH and not their texture. Finally, a mathematical method is presented in chapter 9, which describes a quantitative approach to assess the NOR of soil functioning. Such approach will certainly facilitate and greatly influence future soil quality assessments

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"Eu poderia suportar, embora não sem dor, que tivessem morrido todos os meus amores... mas enlouqueceria se morressem todos os meus amigos!!!"

Vinícius de Moraes

The day I saw Fernando Andreote's e-mail About a Phd position in the Netherlands I couldn't imagine the amazing adventure I was about to face. It was a 4-year phd position in the microbial ecology department, with prof. Dick van Elsas, who he knew and had worked with some time ago.

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List of Publications

1- **Pereira e Silva MC***, Semenov AV, van Elsas JD and Salles JF (2011) Seasonal variations in diversity and abundance of diazotrophic communities across soils. FEMS Microbiology Ecology 77:57-68.

2- **Pereira e Silva MC***, Semenov AV, Heike Schmitt, van Elsas JD and Salles JF (2012) Microbe-mediated processes as indicators to establish the normal operating range of soil functioning. Soil Biology and Biochemistry (In Press).

3- **Pereira e Silva MC***, Poly F, Guillarmaud N, van Elsas JD and Salles JF (2012) Fluctuations in ammonia oxidizer communities are driven by soil structure and pH. Frontiers in Terrestrial Microbiology 3:77, doi: 10.3389/fmicb.2012.00077.

4- **Pereira e Silva MC***, Dias ACF, van Elsas JD and Salles JF (2012) Spatial and temporal variation of archaeal, bacterial and fungal communities in agricultural soils. Plos One, 10.1371/journal.pone.0051554.

5- Semenov AV, **Pereira e Silva MC**, Szturc AE, Schmitt H, Salles JF and van Elsas JD (2012) Impact of incorporated fresh 13C potato tissues on microbial community composition in soil. Soil Biology and Biochemistry 49:88-95.

6- Dias ACF, **Pereira e Silva MC**, Cotta S, Dini-Andreote F, Soares FL, Salles JF, Azevedo JL, van Elsas JD and Andreote FD (2012) Abundance and genetic diversity of nifH gene sequences in anthropogenically affected Brazilian mangrove sediments. Applied and Environmental Microbiology. 78:7960-7967.

7- Procópio da Silva L, **Pereira e Silva MC**, van Elsas JD and Seldin L. Transcriptional profiling of genes involved in n-hexadecane compounds assimilation in the hydrocarbon degrading *Dietzia cinnamea* P4 strain. World Journal of Microbiology (Accepted).

8- Dias ACF, Hoowwout EF, **Pereira e Silva MC**, Salles JF, van Overbeek LS and van Elsas JD (2012) Potato cultivar type affects the structure of ammonia oxidizer communities in field soil under potato beyond the rhizosphere. Soil Biology and Biochemistry 50:85-95.

** Publications from this thesis.*